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THE CHEMICAL CONSTITUTION OF THE PROTEINS

BY

R. H. ADERS PLIMMER, D.Sc.

ASSISTANT PROFESSOR OF PHYSIOLOGICAL CHEMISTRY IN, AND FELLOW OF UNIVERSITY COLLEGE, LONDON

IN TWO PARTS

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TO

EMIL FISCHER

THE MASTER OF

ORGANIC CHEMISTRY IN ITS RELATION TO BIOLOGY

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield

full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

PREFACE.

THE substance Protein, which constitutes the most important part of the material basis of all animal and vegetable life, has naturally attracted the attention and energy of numerous investigators throughout the past century. Progress in the study of this subject, on account of its difficulty, has been exceedingly slow, and it is only of recent years that the discovery of new methods by Emil Fischer has enabled us to increase our knowledge to its present extent. By these methods we have been able to advance from the conception of "albumin" to its actual separation into numerous units, and also to determine their arrangement in the molecule. On this account a monograph embodying the results of the most recent investigations, together with their connections with the work of the other and earlier investigators, needs no excuse for its appearance, as the subject is now being studied in every direction.

On account of the mass of material connected with the subject, this monograph has exceeded the proposed limit in length, and consequently it has become necessary to divide it into two parts:—

I { The Chemical Composition of the Protein Molecule.
  { The Chemical Constitution of its Units.

II. The Synthesis of the Proteins.

R. H. A. P.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS.

PART II.

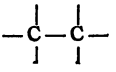
SECTION III.—THE SYNTHESIS OF THE PROTEINS.

Introduction.

IT has been recognised since the time of Liebig that the protein molecule is composed of amino acids, but only during the last decades has it been found that these compounds are so numerous and varied in their chemical composition.

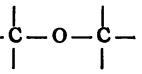
There are various ways in which we can conceive that the amino acids are combined together in the protein molecule. These were summarised and criticised by F. Hofmeister in 1902 as follows:—

I. The carbon atoms can be linked together directly:



Under these conditions the protein molecule would be a huge branched carbon chain, and its degradation into smaller complexes is difficult to explain, and further, such a decomposition by the action of enzymes, *e.g.*, by trypsin, has not yet been observed.

II. The carbon atoms can be linked together by an oxygen atom:

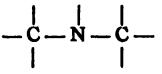


An ether-like combination of the amino acids was suggested by Nasse from the analogy between the hydrolysis of proteins by enzymes and that of the carbohydrates and fats. On account of the small number of hydroxyl groups in the molecules of the amino acids, which is limited to those contained in tyrosine, serine and oxyproline, such a combination can scarcely exist at any rate as the principal method of combination.

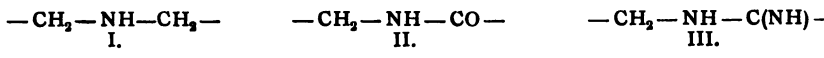
An ester-like combination of the carboxyl group of an amino acid with a hydroxyl group for the same reasons is not possible, nor is the acid anhydride method of combination possible. A further reason

against this mode of combination is the strongly basic character of such compounds, which was first shown by Curtius in the case of glycine ester.

III. The carbon atoms can be linked together by a nitrogen atom :



Several possibilities immediately occur for this mode of combination, of which the three following are the most likely :—



I. II. III.

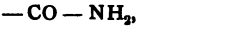
A linking as in Scheme I., which occurs for example in proline, cannot occur to any large extent, since if two amino acids be thus combined together the molecule would become strongly acid in character owing to the free carboxyl groups.

A linking as in Scheme III., which is that of guanidine, occurs in arginine. Only in this compound does such a complex occur in the protein molecule, and therefore such linkings cannot be of the chief importance for the constitution of the molecule.

A large number of important facts support Scheme II. as being the most important for the combination together of the amino acids.

(*a*) The products of hydrolysis.

A small proportion of the total nitrogen of the protein molecule is liberated on hydrolysis as ammonia; this points to the presence of the acid amide,



form of combination.

The greater portion of the total nitrogen—about 90 per cent.—is present in the products of hydrolysis in the form of amino (NH_2) groups, and the remainder in the form of imino (NH) groups, as in arginine.

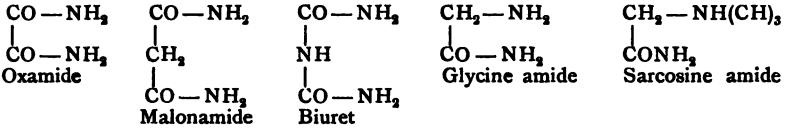
The amino groups are not present in the protein molecule as such, since by the action of nitrous acid on the protein the amount of nitrogen liberated is very small in amount, and in no way corresponds to the amount obtainable if the greater part of the nitrogen be present in the form of amino groups.

It must therefore be assumed that the NH_2 groups of the end products exist in the protein molecule in the form of NH groups.

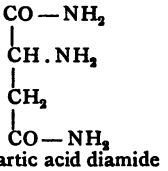
(*b*) The biuret reaction.

The biuret reaction, which is one of the chief characteristics of a

protein, is, according to Schiff, given by those substances which contain two CO — NH complexes, or two CS — NH or C(NH) — NH complexes, and under certain conditions two — CH_2 — NH complexes, combined together directly, or by a carbon atom, or by a nitrogen atom, *e.g.*,

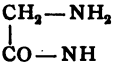


and also

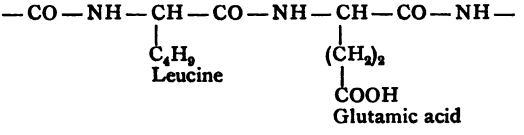


give very intense biuret reactions.

The presence of



groups in the protein molecule is therefore very probable. Such groups occur when amino acids, *e.g.*, leucine and glutamic acid, are combined together in the following way:—



and are repeated when another amino acid is again combined in this manner.

(*c*) The combination of amino acids by the formation of CH_2 — NH — CO groups is also supported by the results obtained in the living body. Hippuric acid $C_6H_5 . CO — NH . CH_2 . COOH$ is formed from benzoic acid and glycine by the kidney, and the bile acids are also combinations of this nature.

(*d*) The various results obtained by the condensation together of amino acids, namely, those by Schaal, Grimaux and Curtius, with his biuret base and hippuric acid compounds (see later), many of which give the biuret reaction, support the above supposition, the proof of which has been given by Emil Fischer by his synthesis of the polypeptides where the group — CO — NH — CH_2 — occurs repeatedly, and is the chief form of combination in the protein molecule.

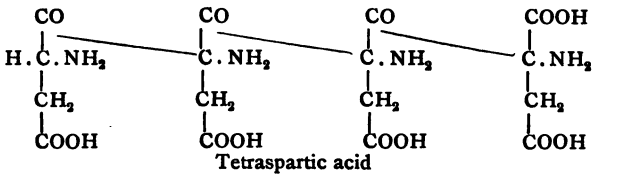
The Condensation Together of Amino Acids.

The earliest investigations upon the condensation together of amino acids were made by Schaal in 1871, who heated asparagine hydrochloride in a current of carbonic acid for three days at 180° C., whereby he obtained a hard white mass, the greater portion of which was insoluble in water and the remainder soluble only with difficulty. The insoluble body was formed by the loss of fifteen molecules of water from eight molecules of aspartic acid, and the other body by the loss of seven molecules of water from four molecules of aspartic acid:—

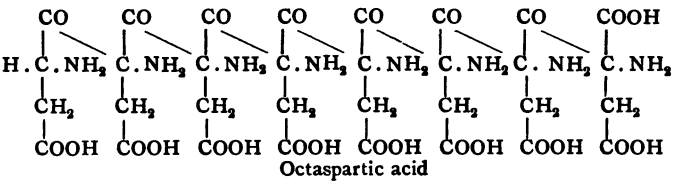
$$C_{16}H_{14}N_4O_9 = 4C_4H_7NO_4 - 7H_2O$$
$$C_{32}H_{26}N_8O_{17} = 8C_4H_7NO_4 - 15H_2O$$

Both compounds were converted into aspartic acid by hydrolysis with baryta water.

J. Guareschi, in 1876, further investigated these substances by determining the amount of silver in the silver salts, but their nature was only demonstrated in 1897-1899 by Schiff. He obtained them by heating aspartic acid, prepared from asparagine and dried at 110° C., for twenty hours at 190-200° C., the yield amounting to 72-75 per cent. Not only were the anhydrides, octaspartide and tetraspartide, as Schiff called these compounds, formed in the process, but also the tetraspartic and octaspartic acids. These acids he also prepared from the anhydrides by hydrolysis with the calculated quantity of cold dilute alkali. From the analysis of their salts, as also their anilides and phenylhydrazides, and from the fact that they gave the biuret reaction which was not observed by Schaal, but pointed out by Grimaux in 1882, he gave these acids the following formulæ:—

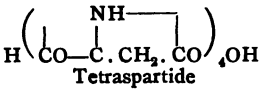


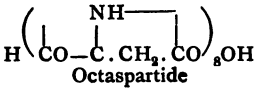
Tetraspartic acid



Octaspartic acid

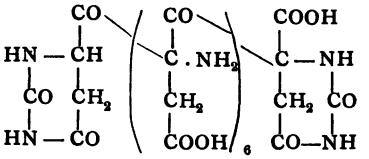
and their anhydrides:—


Tetraspartide


Octaspartide

The octaspartic acid was an octobasic acid, its ninth carboxyl group being neutralised by the adjacent NH_2 group.

The researches of Schutzenberger between 1875 and 1880 upon the products of hydrolysis of proteins by the action of baryta water under pressure, led the French chemists to the belief that the proteins were composed of amino acids and urea or oxamide. In 1882 therefore Grimaux heated Schaal's aspartic acid anhydride with urea for two hours at 125-130° C. A thick mass almost entirely soluble in water resulted; its solution was gelatinous and difficult to filter, and it possessed the properties of colloidal substances, behaving very like albumin. This polyaspartic ureide gave the biuret reaction, and was converted by baryta into carbonic acid, ammonia and aspartic acid; it had the formula $C_{34}H_{40}N_{10}O_{25}$, and consisted of eight molecules of aspartic acid and two molecules of urea. Schiff gave it the formula



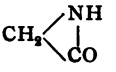
In 1888 Schutzenberger, who regarded proteins to be composed of (1) urea and oxamide; (2) leucines, or amino acids of the formula $C_nH_{2n+1}NO_2$, where n = 6, 5, 4, 3, 2; (3) leuceines, or amino acids of the formula $C_nH_{2n-1}NO_2$, where n = 4, 5, 6, and that there was one molecule of leucine to one molecule of leuceine, prepared the leuceines by the action of ethylene dibromide upon the zinc salts of the lower leucines, such as glycine and alanine, according to the equations

$$C_2H_5NO_2 + C_2H_4Br_2 = 2HBr + C_4H_7NO_2$$
$$C_3H_7NO_2 + C_2H_4Br_2 = 2HBr + C_5H_9NO_2$$

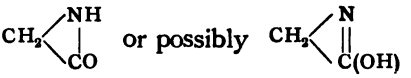
and in 1891 heated a mixture of leucine and leuceine with 10 per cent. urea, carefully dried at 110°, with phosphoric anhydride. He obtained a mass soluble in water, which was precipitated by several volumes of alcohol; it gave the biuret reaction and other protein reactions, and Schutzenberger regarded it as a "pseudo-peptone synthetique".

The Biuret Base.

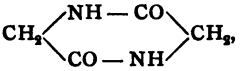
In 1883 Curtius first prepared glycine ester by decomposing glycine ester hydrochloride with silver oxide. It was a colourless, strongly basic oil, very unstable, and only capable of preservation in dry ether. If it were allowed to stand in the air, it underwent decomposition and was converted into an insoluble anhydride,



and a soluble base, which gave the biuret reaction and was called the biuret base. Further investigations upon the nature of these compounds were made by Curtius and Goebel in 1888, who found that the glycine anhydride separated when the ester was allowed to stand for a few days with four volumes of water, and that from the analysis of its silver and copper compounds it had the formula

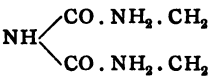


No result could be arrived at concerning the biuret base, which was prepared by keeping pure glycine ester in a sealed tube, when it changed into a white, crystalline mass. Curtius and Schulze, in 1890, by molecular weight determinations found that the formula of the anhydride must be doubled, and it was probably represented by



which was ultimately proved by Fischer and Fourneau in 1901.

This anhydride or biuret base was investigated again in 1894 by Lilienfeld, who prepared it by heating glycine ester with solid potassium bisulphate on the water bath, and who gave it the formula $C_4H_9N_3O_2$, and the constitution



since he also obtained dimethylamine, ethyl ether and carbonic acid in its preparation.

When he heated it with water, Lilienfeld obtained a flocculent precipitate, just as Curtius and Goebel had observed; this formed a gelatinous mass after filtering which contracted like gelatin and behaved, in fact, very like glutin. In a similar manner Lilienfeld condensed

leucine ester and tyrosine ester with glycine ester, whereby he obtained a peptone-like body giving all the principal protein reactions.

Another anhydride of glycine was obtained in 1900 by Balbiano and Trasciatti who heated glycocoll with glycerol; it was a yellowish powder insoluble in all neutral solvents, like the horny substances, and on hydrolysis Balbiano found that it was reconverted into glycine.

Not, however, until 1904 were any further investigations carried out concerning the constitution of the biuret base. Schwarzschild then suggested that it consisted of seven glycine molecules combined together in an open chain, and that it was the ethyl ester of hexaglycyl-glycine of the formula

$$NH_2 . CH_2 . CO — (NH . CH_2 . CO)_5 — NH . CH_2 . COOC_2H_5,$$

but Curtius shortly afterwards showed that this was erroneous, and that the body with which Schwarzschild had worked was still a mixture of glycine anhydride and biuret base.

By studying the conditions under which glycine ester was converted into glycine anhydride and biuret base, Curtius showed that, when moisture was excluded as completely as possible, the biuret base with only traces of glycine anhydride was formed, and that the amount of glycine anhydride produced increased with the amount of water present. Thus, if pure glycine ester were kept in the absence of air, it solidified in a few days and the mass contained biuret base with 23-24 per cent. of glycine anhydride; if glycine ester were boiled with dry chloroform, 12 per cent. of glycine anhydride was formed, but if perfectly pure glycine ester were mixed with about a third of its volume of absolute ether and left for some weeks it was almost completely converted into biuret base, only 1 per cent. of glycine anhydride being present. The analysis, molecular weight, properties and reactions of the biuret base showed conclusively that it was triglycyl-glycine ester, *i.e.*, a tetraglycyl compound of the formula

$$NH_2 . CH_2 . CO — NH . CH_2 . CO — NH . CH_2 . CO — NH . CH_2 . COOC_2H_5.$$

It was observed by Curtius and Gumlich that the biuret base when heated *in vacuo* to 100° C. lost alcohol, and that it was converted into an anhydride, most probably octoglycine anhydride, so that from glycine ester quite complex substances can be obtained. Such substances have also been obtained by Emil Fischer and his pupils by the condensation of amino acid and polypeptide esters (see below).

The Linking Together of Amino Acids.

By the action of benzoyl chloride upon the silver salt of glycine, Curtius, in 1881, obtained in addition to the expected hippuric acid

$$C_6H_5CO.Cl + H_2N.CH_2.COOH = HCl + C_6H_5.CO.NH.CH_2.COOH$$

two other acids of higher molecular weight. One of these was hippuryl-glycine or benzoyl-glycyl-glycine,

$$C_6H_5CO.HN.CH_2.CO—NH.CH_2.COOH,$$

as was proved by the study of its salts, its ethyl ester and amide, and by its hydrolysis into hippuric acid and glycine. It was the first definite compound known which contained two amino acid residues combined together.

The constitution of the other acid, called the γ-acid, could not be determined; it had the formula $C_{10}H_{12}N_3O_4$, was soluble with difficulty in water and gave the biuret reaction. It was formed in larger quantities when hippuric ester was fused with glycine, but under these conditions another compound—benzoyl-bisglycyl-glycine—was formed at the same time, so that in this way a series of compounds resulted, each succeeding member containing a glycyl- $NH_2.CH_2.CO$- group more than the preceding one.

Further investigations upon the constitution of these compounds were only carried out in 1904 by Curtius and Benrath, who found that the γ-acid from the analysis of its silver salt, ester, etc., had the formula $C_{19}H_{24}N_6O_8$, and that it was benzoyl-pentaglycyl-glycine,

$$C_6H_5.CO—(NH.CH_2.CO)_5—NH.CH_2.COOH,$$

its ester being identical with the compound synthesised by Curtius and Wüstenfeld (see below).

Two other compounds—hippuryl-glycine and benzoyl-triglycyl-glycine—were found to be formed by fusing together hippuric ester and glycine, but not the previously isolated benzoyl-diglycyl-glycine. Longer chains than the six membered γ-acid are not believed by Curtius and Benrath to be formed in this reaction.

In 1890 Curtius, by the action of hydrazine upon benzoyl chloride, benzamide or benzoic ester, obtained benzoylhydrazine,

$$C_6H_5.COOC_2H_5 + H_2N.NH_2 = C_6H_5.CO.HN.NH_2 + HOC_2H_5,$$

which, when treated with nitrous acid, gave benzoylazoimide,

$$C_6H_5.CO.HN.NH_2 + HNO_2 = 2H_2O + C_6H_5.CO—N\begin{matrix}N\\ \|\|\\ N\end{matrix}$$

By combining benzoylazoimide with glycine, he synthesised hippuric acid,

$$C_6H_5 . CO - N\langle \overset{N}{\underset{N}{\|}} + H_2N . CH_2 . COOH = C_6H_5 . CO . NH . CH_2 . COOH + N_3H,$$

and by carrying out the same series of reactions with hippuric acid, the compound hippurazide or hippurylazoimide was obtained, which could be employed in synthesis in the place of the unknown hippuryl chloride; thus from hippuric ester by the following series of reactions hippuryl-glycine was obtained identical with the compound previously obtained in 1883 by the action of benzoyl chloride upon the silver salt of glycine :—

$$\underset{\text{Hippuric ester}}{C_6H_5 . CO . NH . CH_2 . COOC_2H_5} + \underset{\text{Hydrazine}}{H_2N . NH_2} = \underset{\text{Hippuryl-hydrazine}}{C_6H_5 . CO . NH . CH_2 . CO . HN . NH_2} + HOC_2H_5$$

$$\underset{\text{Hippuryl-hydrazine}}{C_6H_5 . CO . NH . CH_2 . CO . HN . NH_2} + HNO_2 = \underset{\text{Hippurazide}}{C_6H_5 . CO . NH . CH_2 . CO - N\langle \overset{N}{\underset{N}{\|}}}$$

$$\underset{\text{Hippurazide}}{C_6H_5 . CO . NH . CH_2 . CO - N\langle \overset{N}{\underset{N}{\|}}} + \underset{\text{Glycine}}{H_2N . CH_2 . COOH} =$$

$$\underset{\text{Hippuryl-glycine}}{C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . COOH} + N_3H$$

This method of combining together amino acids was further extended by Curtius and Wüstenfeld in 1902 by preparing the ester, the hydrazide and azide of this compound and again combining it with glycine, when benzoyl-diglycyl-glycine was obtained :—

$$C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . COOH \rightarrow$$
$$C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . COOC_2H_5 \rightarrow$$
$$C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . CO - NH . NH_2 \rightarrow$$
$$C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . CO - N\langle \overset{N}{\underset{N}{\|}} \rightarrow$$
$$C_6H_5 . CO . NH . CH_2 . CO - NH . CH_2 . CO - NH . CH_2 . COOH$$

From this compound benzoyl-triglycyl-glycine was prepared by the same series of reactions, and benzoyl-tetraglycyl-glycine by continuing the process.

Further experiments upon the formation of glycyl chains with hippurazide were carried out by Curtius and Levy; by combining hippurazide with glycyl-glycine ester, prepared by the method of Fischer and Fourneau (see below), benzoyl-diglycyl-glycine ester, identical with the compound prepared by Curtius and Wüstenfeld, was obtained. The azide of this compound when combined with glycyl-glycine hydrochloride gave benzoyl-tetraglycyl-glycine :—

$$C_6H_5 . CO . (NH . CH_2 . CO)_4 . NH . CH_2 . COOH.$$

This compound was also prepared from benzoyl-triglycyl-glycine azide and glycine ester.

The further lengthening of the chain by means of the azide of benzoyl-tetraglycyl-glycine could not be accomplished since this compound could not be prepared, but the next member of the series, benzoyl-pentaglycyl-glycine ester,

$$C_6H_5 . CO . (NH . CH_2 . CO)_5 . NH . CH_2 . COOC_2H_5,$$

was prepared from benzoyl-triglycyl-glycine azide and glycyl-glycine ester. This was identical with the original γ-acid of 1883, of which Curtius and Benrath had determined the constitution.

By condensing the biuret base, which in the meanwhile had been proved to be triglycyl-glycine ester with hippurazide, Curtius and Levy obtained again the former benzoyl-tetraglycyl-glycine, and by condensing it with hippuryl-glycine azide they obtained benzoyl-pentaglycyl-glycine ester, and thus by a less circuitous method attained to the same compound as they had prepared from hippurazide. Further lengthening of the glycyl chain has not as yet been carried out by this method, but the method has been adapted by Curtius and Lambotte to the formation of alanine chains, namely:—

Hippuryl-alanine, $C_6H_5 . CO . NH . CH_2 . CO — NH . CH(CH_3) . COOH$ from hippurazide and α-alanine.

Hippuryl-alanyl-alanine, $C_6H_5 . CO . NH . CH_2 . CO — NH . CH(CH_3) . CO — NH . CH(CH_3) . COOH$ from hippuryl-alanine azide and α-alanine.

Hippuryl-alanyl-alanyl-alanine, $C_6H_5 . CO . NH . CH_2 . CO — NH . CH(CH_3) . CO — NH . CH . (CH_3) . CO — NH . CH(CH_3) . COOH$ from hippuryl-alanyl-alanine azide and α-alanine.

These all contain the glycine residue as well as the alanine residue in their molecule; in order to eliminate the glycine residue and obtain compounds without the glycine radical, Curtius and van der Linden prepared the following compound:—

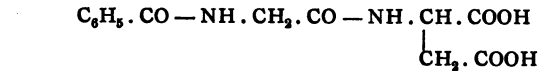
Benzoyl-alanyl-alanine, $C_6H_5 . CO . NH . CH(CH_3) . CO — NH . CH(CH_3) . COOH$ from benzoyl-alanine azide, which was prepared from benzoyl-alanine, as obtained by Fischer's method, and alanine.

Benzoyl-alanine azide was also combined with glycine, and this radical introduced at the end of the chain; thus they prepared

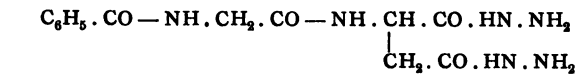
Benzoyl-alanyl-glycine, $C_6H_5 . CO . NH . CH(CH_3) . CO — NH . CH_2 . COOH$ from benzoyl-alanine azide and glycine.

Benzoyl-alanyl-glycyl-glycine, $C_6H_5 . CO . NH . CH(CH_3) . CO — NH . CH_2 . CO — NH . CH_2 . COOH$ from benzoyl-alanyl-glycine azide and glycine, or benzoyl-alanine azide and glycyl-glycine.

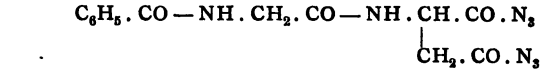
The behaviour of hippurazide with the dibasic aspartic acid was investigated by Th. and H. Curtius in order to build up chains containing this amino acid. By the action of hippurazide upon aspartic acid in alkaline solution they obtained hippuryl-aspartic acid:—



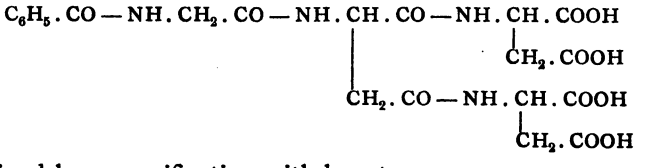
The ester of this compound was converted into the hydrazide by means of hydrazine,



from which hippuryl-aspartic acid azide was obtained by the action of nitrous acid :—

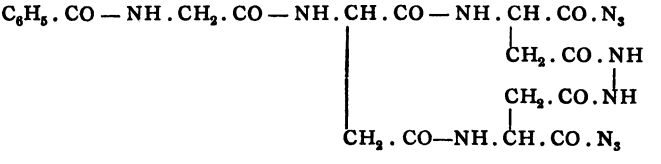


This reacted in ethereal solution with aspartic ester yielding hippuryl-asparagyl-aspartic ester from which the free acid,

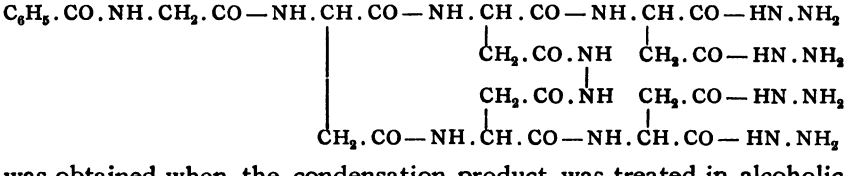


was obtained by saponification with baryta.

The hydrazide of this compound was then prepared from the ester in the usual manner, and from this the azide, which did not, however, possess the normal structure, but that of the hydrazi-azide,

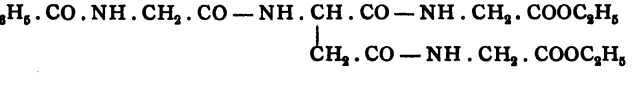


The condensation product of this compound with aspartic ester was not isolated, but the complex hippuryl-disaspartyl-aspartic acid hydrazi-hydrazide, its hydrazine derivative,



was obtained when the condensation product was treated in alcoholic solution with hydrazine hydrate.

Just in the same way hippuryl-aspartyl-glycine ester,



resulted when hippuryl-aspartic acid azide was combined with glycine ester.

In conjunction with Gumlich, Curtius has investigated the linking of hippurazide with β-amino-α-oxypropionic acid and with β-amino-butyric acid. With the former compound, the combination took place with the hydroxyl group instead of with the amino group, hippuryl-α-oxy-β-aminopropionic acid,

$$C_6H_5 . CO . NH . CH_2 . CO — O — CH . (CH_2 . NH_2) . COOH,$$

being formed. With the latter, by the usual series of reactions they prepared hippuryl-β-aminobutyric acid and hippuryl-β-aminobutyryl-β-aminobutyric acid.

Curtius and Müller have also prepared hippuryl-γ-aminobutyric acid and hippuryl-β-phenyl-α-alanine, compounds of no great interest since these amino acids do not occur in the protein molecule. They show, however, that not only can α-amino acids be combined together by the azide method, but also β- and γ- substituted amino acids.

In order to build up chains containing the carbamic acid radical, NH . CO, just as Curtius and his co-workers have built up chains containing glycyl, NH . CH_2 . CO, alanyl and asparagyl radicals, Curtius and Lenhard, in 1904, proposed to make use of the azide of hippuryl-carbamic acid,

$$C_6H_5 . CO . NH . CH_2 . CO — NH . CO . N_3.$$

This compound, however, was unavailable, since sufficient quantities of hippuryl urea, which Curtius had formerly prepared from hippuric ester and urea, could not be obtained from hippurazide and urea. They therefore attempted to make the azide of benzoylcarbamic acid by the action of hydrazine on benzoylurea, but the only product which they obtained was the hydrazide of benzoic acid. The benzoic acid radical is therefore very easily eliminated from the urea molecule, the molecule of benzoylcarbamic acid hydrazide, being hydrolysed according to the equation

$$C_6H_5 . CO . NH . CO . NH . NH_2 + H_2O = C_6H_5 . CO . NH . NH_2 + NH_3 + CO_2.$$

This non-success led them to attempt to combine phenylcarbamic acid azide $C_6H_5 . NH . CO — N_3$, which Curtius and Hofmann and Curtius and Burkhardt had described in 1896 and 1898, with urea, but again the desired result was not achieved, nor could a combination of this compound with biuret be effected.

It followed therefore that acid radicals cannot be combined with urea by the acid azide reaction.

If, however, glycine were used instead of urea for combination with the azide of phenylcarbamic acid phenylcarbaminoglycine resulted, which was identical with the compound prepared by Paal in 1894 from phenylisocyanate and glycine.

With this compound Curtius and Lenhard continued the lengthening of the chain by the azide reaction, and obtained

Phenylcarbaminoglycyl-glycine, $C_6H_5 . NH . CO—NH . CH_2 . CO—NH . CH_2 . COOH$, and phenylcarbaminodiglycyl-glycine, $C_6H_5 . NH . CO—NH . CH_2 . CO—NH . CH_2 . CO—NH . CH_2 . COOH$.

These compounds and their various derivatives prepared by Curtius and his pupils are white crystalline compounds, for|the most part soluble with difficulty in cold water. Some of them give the biuret reaction, but others do not, in particular the less complex compounds where the influence of the acid radical inhibits the reaction, although the compounds possess the exact conditions, as determined by Schiff, for the positive exhibition of the reaction.

The reactions given by the azides with alcohol, ammonia, aniline, etc., are of greater interest and may therefore be briefly summarised.

By the action of ammonia the acid azides are either completely saponified into the corresponding acids, or by a rearrangement in the molecule, they are converted into derivatives of urea. With the dibasic acids both possibilities may occur at the same time, and the resulting compound is half acid amide and half urethane. Subsequent hydrolysis clearly shows the nature of the component amino acid chain. Thus, hippuryl urethane is formed from hippurazide and alcohol,

$$C_6H_5 . CO . NH . CH_2 . CO . N_3 + C_2H_5OH = C_6H_5 . CO . NH . CH_2 . NH . COOC_2H_5 + N_2,$$

and on hydrolysis it is converted into benzoic acid, ammonia, carbonic acid and formaldehyde:—

$$C_6H_5 . CO . NH . CH_2 . NH . COOC_2H_5 + 3H_2O = C_6H_5 . COOH + 2NH_3 + HCHO + CO_2 + C_2H_5OH.$$

The reaction therefore leads to the formation of formaldehyde from glycine. Hippuryl alanineazide and aniline give the following urea derivative:—

$$C_6H_5 . CO . NH . CH_2 . CO—NH . CH(CH_3) . CO—NH . C_6H_5,$$

which, on hydrolysis, breaks down into hippuric acid, ammonia, acetaldehyde, carbonic acid and aniline:—

$$C_6H_5 . CO . NH . CH_2 . CO—NH . CH(CH_3) . CO—NH . C_6H_5 + 3H_2O = C_6H_5 . CO . NH . CH_2 . COOH + 2NH_3 + CH_3 . CHO + CO_2 + H_2N . C_6H_5.$$

The same products are obtained when the urethane derivative, obtained from benzoylalanine azide and alcohol, is hydrolysed:

$$C_6H_5 . CO . NH . CH . (CH_3) . NH . COOC_2H_5 + 3H_2O = C_6H_5 . COOH + 2NH_3 + CH_3CHO + CO_2 + C_2H_5OH,$$

except that benzoic acid appears in the place of hippuric acid. Hippuryl aspartic acid azide and aniline give a compound which is half anilide and half carbanilide, and this on hydrolysis is converted into α-β-diaminopropionic acid, hippuric acid, aniline and carbonic acid :—

$$\begin{aligned} &C_6H_5.CO.NH.CH_2.CO-NH.CH.CO-NH.C_6H_5 \\ &\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ &\qquad\qquad\qquad\qquad\qquad\qquad CH_2.NH.CO.NH.C_6H_5 \end{aligned} + 3H_2O =$$

$$\begin{aligned} &C_6H_5.CO.NH.CH_2.COOH + NH_2.CH.COOH \\ &\qquad\qquad\qquad\qquad\qquad\quad | \\ &\qquad\qquad\qquad\qquad\qquad CH_2.NH_2 \end{aligned} + 2C_6H_5.NH_2 + CO_2$$

The normal urethane formed by the action of alcohol on hippuryl aspartic acid azide, yields on hydrolysis hippuric acid, carbonic acid, alcohol and aminoacetaldehyde :—

$$\begin{aligned} &C_6H_5.CO.NH.CH_2.CO-NH.CH.NH.COOC_2H_5 \\ &\qquad\qquad\qquad\qquad\qquad\quad | \\ &\qquad\qquad\qquad\qquad\qquad CH_2.NH.COOC_2H_5 \end{aligned} + 4H_2O =$$

$$C_6H_5.CO.NH.CH_2.COOH + 2NH_3 + NH_2.CH_2.CHO + CO_2 + 2C_2H_5OH$$

The first reaction shows the conversion of a compound belonging to the series of dibasic monoamino acids into a diaminomonocarboxylic acid ; in the second reaction, a dibasic amino acid is changed into the aldehyde of the monobasic glycine.

Finally, propylenediamine was obtained when the urethane, resulting from the action of alcohol upon hippuryl-β-aminobutyric acid azide, was hydrolysed :—

$$\begin{aligned} &C_6H_5.CO.NH.CH_2.CO-NH.CH(CH_3).CH_2.NH.COOC_2H_5 + 3H_2O = \\ &C_6H_5.COOH + NH_2.CH_2.COOH + NH_2.CH(CH_3).CH_2.NH_2 + CO_2 + C_2H_5OH \end{aligned}$$

which shows the conversion of an amino acid derivative into a diacid base.

These transformations of amino acid derivatives increases our interest in these compounds prepared by Curtius and his pupils, and gives an impulse to their further study, especially as formaldehyde is such an important compound in the synthesis of sugars by plants, and as the diamino acids and diamines occur as products of decomposition of proteins by enzymes and bacteria, although according to our present knowledge they are not formed in nature in this manner.

These compounds have, however, given us an insight into complex glycine, alanine and aspartic acid derivatives. E. Fischer has prepared by his methods (see under polypeptides) compounds containing these amino acids without the presence of the benzoyl group which is strange to the protein molecule, but at present the aspartic acid compounds, if we disregard Schiff's polyaspartic acid, which probably has another constitution than that represented, are the most complex substances known containing this important constituent of the protein molecule.

The further work, published in 1906, of Curtius and his pupils is concerned with the action of nitrous acid upon the polyglycine compounds: diazoacetyl glycine ester $N_2 : CH . CO — NH . CH_2 . COOC_2H_5$ was formed by the action of nitrous acid upon glycyl-glycine ester hydrochloride and diazoacetyl glycyl-glycine ester $N_2 : CH . CO — NH . CH_2 . CO — NH . CH_2 . COOC_2H_5$ by its action upon diglycyl-glycine ester hydrochloride.

By the action of ammonia on the first body diazoacetyl glycine amide,

$$N_2 : CH . CO — NH . CH_2 . CO . NH_2,$$

was formed, and on the latter diazoacetyl glycyl-glycine amide,

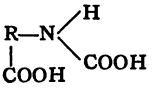
$$N_2 : CH . CO — NH . CH_2 . CO — NH . CH_2 . CO — NH_2.$$

These are yellow substances, but when excess of ammonium hydroxide or liquid ammonia is added to the esters colourless substances are obtained; they were at first regarded as azomethane derivatives, but have since been shown to be the ammonium salts, *e.g.*, of isodiazoacetyl glycine amide,

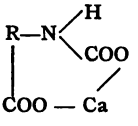
$$\begin{matrix} & N \\ & \| \\ NH_4 . N & \diagdown C . CO . NH . CH_2 . CO . NH_2. \end{matrix}$$

Combinations of Amino Acids with Carbonic Acid.

The sodium and barium salts of the monoamino acids have a strongly alkaline reaction and are highly dissociated salts. If carbonic acid be passed into the solution of the barium salt, barium carbonate is not, as would be expected, immediately formed; the solution remains clear, and only after a short time, when the solution becomes saturated with carbonic acid, does it become cloudy and barium carbonate gradually separates out; the separation of barium carbonate is hastened by heating. This phenomenon is due, as was shown by Siegfried in 1905, to the formation of salts of carbamino acids of the general formula



i.e., to the formation of a dibasic acid of which the calcium salt,



is soluble with difficulty in ice-cold water and alcohol. Similar compounds are formed with the dibasic aspartic and glutamic acids and with the diamino acids. In aqueous solutions also the free carbamino acid is formed. The reaction may serve, as Siegfried pointed out in 1906, for the separation of amino acids from their solutions.

Siegfried and Neumann, in 1908, showed that there was a distinct regularity in the fixation of carbonic acid by amino acids; the amino groups of the aliphatic amino acids were quantitatively converted into carbamino groups; in histidine and arginine only the amino group of the side chain, not the nitrogen atoms of the rings, reacted with carbonic acid to form carbamino groups.

Glycyl-glycine was also found by Siegfried to react with carbonic acid in the presence of barium hydrate with the formation of the barium salt of glycyl-glycine carbamino acid, which on heating was converted into barium carbonate and glycyl-glycine. Further, Siegfried and Liebermann have shown that the peptide linking in the polypeptides reacts to a certain extent, and by this means they hope to obtain an idea of the constitution of the various peptones which Siegfried has isolated from proteins by the action of trypsin.

Not only do the amino acids react with carbonic acid in the presence of calcium salts, but also peptones and the proteins of serum; this may

explain certain of the phenomena concerning the presence of carbonic acid in blood and in working muscle; a protein carbonic acid compound may be formed which can give rise to carbonic acid without taking up oxygen.

Siegfried's results with glycine and glycyl-glycine have been confirmed by Leuchs, who, in addition, has investigated the combinations of amino acids and of polypeptides with carbonic acid which were prepared by Fischer and his pupils by combination with chlorocarbonic ester (see later).

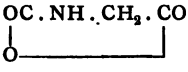
Carbethoxylglycine which was obtained by combining together chlorocarbonic ester with glycine,

$$Cl.COOC_2H_5 + H_2N.CH_2.COOH = HCl + C_2H_5.O.OC.NH.CH_2COOH,$$

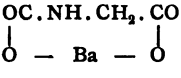
even by careful hydrolysis could not be converted into the free acid, decomposition always occurring with the formation of glycine and carbonic acid; Leuchs, however, in 1907, obtained the free acid indirectly in the following manner: Carbethoxylglycine was converted into its acid chloride,

$$C_2H_5.O.OC.NH.CH_2.COCl,$$

by the action of thionylchloride, and this compound, when heated, lost ethyl chloride and was changed into the anhydride,



which, when warmed with water to 15° C., decomposed into glycine and carbonic acid, but, when treated with the calculated quantity of baryta, yielded the barium salt of glycine carboxylic acid,

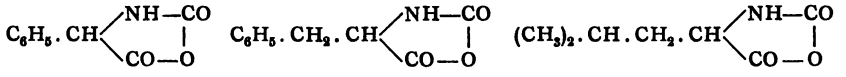


This was identical with the barium salt obtained by Siegfried from glycine, carbonic acid and barium hydrate.

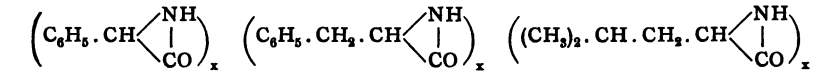
It is of interest to observe that Leuchs found that the anhydride, when treated with a small quantity of water, gave an anhydride of glycine which was not identical with diketopiperazine, but possibly the same substance which Balbiano and Trasciatti (p. 7) obtained by heating glycine with glycerol, or as that obtained by Curtius from the biuret base (p. 7).

Leuchs and Geiger, in 1908, obtained the anhydrides of C-phenylaminoacetic acid, of phenylalanine and of leucine in the same way by heating the acid chlorides of the carbomethoxyl derivatives, which were

prepared by the action of thionyl chloride, whereby methyl chloride was eliminated,



and then warming in the presence of traces of water, when carbon dioxide was evolved with the formation of the anhydrides,



Carbethoxyl-glycyl-glycine ester was found by Fischer to yield on hydrolysis the free glycyl-glycine carboxylic acid, from which, on esterification, an ester was obtained, which was isomeric with the original carbethoxyl-glycyl-glycine ester. This acid was extremely stable in comparison with the glycyl-glycine-N-carboxylic acid obtained by Siegfried in 1906 and also by Leuchs.

The difference between these compounds was shown by Leuchs and Manasse, in 1907, to be due to the fact that the original ester, which has the lactam formula and belongs to the *a*-series,

$$C_2H_5.O.OC.NH.CH_2.CO.NH.CH_2.COOC_2H_5,$$

undergoes a transformation during hydrolysis and is converted into the acid having the lactim formula and belonging to the *β*-series,

$$C_2H_5.O.OC.NH.CH_2.C(OH)=N.CH_2.COOC_2H_5.$$

This was proved by the study of the phenyl derivatives,

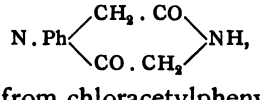
Carbethoxyl-glycyl-N-phenyl-glycine ester $C_2H_5.O.OC.NH.CH_2.CO.N(C_6H_5).CH_2.COOC_2H_5$, and carbethoxyl-N-phenylglycyl-glycine ester $C_2H_5.O.OC.N(C_6H_5).CH_2.CO.NH.CH_2.COOC_2H_5$.

The former was prepared from carbethoxyl-glycyl chloride and phenylglycine ester, the latter from carbethoxyl-N-phenylglycyl chloride and glycine ester.

Owing to the substitution of the hydrogen atom by phenyl in the position represented in carbethoxyl-glycyl-N-phenyl-glycine ester, no transformation into the lactim form can take place. On hydrolysis it yielded the dipeptide glycyl-phenylglycine, with loss of carbonic acid,

$$NH_2.CH_2.CO.N(C_6H_5).CH_2.COOH,$$

and this dipeptide was converted on heating into the diketopiperazine,



which was also obtained from chloracetylphenylglycine and ammonia.

Carbethoxyl-N-phenyl-glycyl-glycine ester on hydrolysis yielded the acid

$$HOOC.N(C_6H_5).CH_2.C(OH)=N.CH_2.COOH,$$

which did not lose carbon dioxide, and was analogous to Fischer's glycyl-glycine carboxylic acid. Phenyl-glycyl-glycine carboxylic acid easily forms the lactone

$$N(C_6H_5)\begin{<}CH_2\\CO.O\end{>}C=N.CH_2.COOH,$$

as do also its derivatives; thus carbethoxyl-N-phenyl-glycyl-glycine ester when treated with ammonia yields the amide

$$C_2H_5O.OC.N(C_6H_5).CH_2.C(OH)=N.CH_2.CO.NH_2,$$

which loses alcohol at 220° and forms the lactone

$$N(C_6H_5)\begin{<}CH_2\\CO.O\end{>}C=N.CH_2.CO.NH_2.$$

The stability of glycyl-glycine carboxylic acid obtained from carbethoxyl-glycyl-glycine ester is therefore due to its having the lactim formula,

$$HOOC.NH.CH_2.C(OH)=N.CH_2.COOH,$$

whereas the instability of glycyl-glycine carboxylic acid obtained from glycyl-glycine and carbonic acid is due to the lactam formula,

$$HOOC.NH.CH_2.CO.NH.CH_2.COOH.$$

Further proof for these formulæ is given by Siegfried's experiments in which he showed that the peptide linking in polypeptides, which have the lactam formula, could also combine with carbonic acid, whereas glycyl-glycine carboxylic acid which has the lactim formula did not combine with carbonic acid.

Combinations of Amino Acids with Ammonia.

The amides of amino acids, such as glycinamide,

$$NH_2 . CH_2 . CO — NH_2,$$

the compounds prepared by Curtius, and the polypeptides (later) of Emil Fischer, such as glycyl-glycine,

$$NH_2 . CH_2 . CO — NH — CH_2 . COOH,$$

are combinations of amino acids in which one carboxyl group only is attached to the ammonia residue.

A compound, in which two carboxyl groups of amino acids are attached to ammonia, namely, diglycinimide,

$$NH_2 . CH_2 . CO — NH — OC . CH_2 . NH_2,$$

was prepared in 1907 by Bergell. Starting from chloracetamide he obtained chloracetonitrile by heating it with phosphoric anhydride :—

$$Cl . CH_2 . CO . NH_2 = H_2O + Cl . CH_2 . CN.$$

From this compound dichlorodiacetamide resulted in its interaction with chloracetic acid,

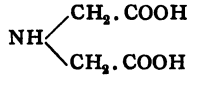
$$Cl . CH_2 . CN + HOOC . CH_2Cl = Cl . CH_2 . CO — NH — OC . CH_2Cl,$$

and on treating it with ammonia, diglycinimide hydrochloride was obtained,

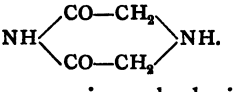
$$Cl . CH_2 . CO — NH — OC . CH_2Cl + 3NH_3 =$$
$$NH_4Cl + NH_2 . CH_2 . CO — NH — OC . CH_2 . NH_2 + HCl,$$

from which the free base was prepared by means of silver oxide as a crystalline substance of a basic character.

Bergell continued his work on this substance in conjunction with Feigl. Diglycinimide was stable to acids and to the weak alkalies, magnesia and sodium bicarbonate, but it was converted by caustic alkalies and by baryta into ammonia and an acid of the constitution



which was identical with "diglykolamidsäure" prepared in 1862 by Heintz. Its formation probably took place through the intermediate ring compound,



It did not give rise to ammonia and glycine as was expected; its benzoyl derivative, however, on hydrolysis was converted into hippuric acid, ammonia and glycine :—

$$C_6H_5 . CO . NH . CH_2 . CO — NH — OC . CH_2 . NH_2 + 2H_2O =$$
$$C_6H_5 . CO . NH . CH_2 . COOH + NH_3 + HOOC . CH_2 . NH_2.$$

In order to introduce another glycyl residue into diglycinimide, the chloracetyl derivative was prepared, from which by the action of ammonia glycyl-diglycinimide,

$$NH_2 . CH_2 . CO . NH . CH_2 . CO — NH — OC . CH_2 . NH_2,$$

did not result; but ammonia was lost and a compound probably of the formula

$$\begin{array}{l} CH_2 . CO . NH . CH_2 . CO—NH—OC . CH_2 . NH \\ \quad\quad\quad\

The Polypeptides.

Our knowledge of the structure of the protein molecule has been given us by the systematic researches of Emil Fischer and his pupils which were commenced in 1901, the combinations together of the amino acids being termed the polypeptides. This designation is in imitation of that of the carbohydrates, where we differentiate between mono-, di-, tri-, poly-saccharides, and it retains the word peptone, on account of the very similar properties of these substances to peptone, which most probably consists of a mixture of polypeptides.

Three general methods have been devised for the synthesis of the polypeptides which are best described separately.

Method I. Synthesis from the Esters.

The ester of glycine was first prepared, as previously mentioned, by Curtius in 1883, and it was observed by Curtius and Goebel, in 1888, that the ester lost alcohol and was converted into 2, 5-diketo- or diacipiperazine.

Similar compounds—leucinimide and lactimide—had been obtained from leucine and alanine. These anhydrides form the starting-point in the synthesis of the polypeptides by this method, and they are best obtained by heating the ester of the amino acids in a sealed tube to 150-180° C. for some hours.

Fischer and Fourneau, in 1901, found that 2, 5-diketopiperazine, or glycine anhydride, as it is now best termed, was converted by boiling with concentrated hydrochloric acid into the hydrochloride of an amino acid of the formula $C_4H_8N_2O_3$, from which they obtained the free acid by treatment with the calculated quantity of caustic soda, or by means of silver oxide. Its formation is represented by the equation

$$\begin{matrix} NH-CH_2-CO \\ | \qquad\quad | \\ CO-CH_2-NH \end{matrix} + H_2O = NH_2.CH_2.CO-NH.CH_2.COOH.$$

The compound is the first anhydride of glycine, and was termed glycyl-glycine, the group $NH_2.CH_2.CO$ being called the glycyl group.

By treating glycine anhydride with alcoholic hydrochloric acid, glycyl-glycine ester resulted :—

$$\begin{matrix} NH-CH_2-CO \\ | \qquad\quad | \\ CO-CH_2-NH \end{matrix} + C_2H_5OH = NH_2.CH_2.CO-NH.CH_2.COOC_2H_5.$$

Both the free acid and its ester have a great tendency to become reconverted into glycine anhydride, and both compounds are characterised by

the great reactiveness of the NH_2 group; thus, with phenylisocyanate they both yield the compound $C_6H_5 . NH . CO . NH . CH_2 . CO . NH . CH_2 . COOH$, and the ester gives with ethyl chlorocarbonate, carbethoxyl-glycyl-glycine ester :—

$$\begin{matrix} OC_2H_5 \\ \diagup \\ C=O \\ \diagdown \\ Cl \end{matrix} + NH_2 . CH_2 . CO . NH . CH_2 . COOC_2H_5 = C_2H_5 . O . OC . NH . CH_2 . CO . NH . CH_2 . COOC_2H_5,$$

from which the amide

$$C_2H_5 . O . OC . NH . CH_2 . CO . NH . CH_2 . CONH_2$$

is obtained by the action of ammonia, and the free acid

$$C_2H_5 . O . OC . NH . CH_2 . CO . NH . CH_2 . COOH$$

by careful hydrolysis with soda.

Carbethoxyl-glycyl-glycine ester, when heated with leucine ester, yielded carbethoxyl-glycyl-glycyl-leucine ester,

$$C_2H_5O . OC . NH . CH_2 . CO . NH . CH_2 . COOC_2H_5 + NH_2 . CH(C_4H_9) . COOC_2H_5 = C_2H_5OH + C_2H_5O . OC . NH . CH_2 . CO . NH . CH_2 . CO . NH . CH(C_4H_9) . COOC_2H_5,$$

a compound which contains three amino acids combined together and was the first known representative of a tripeptide.

Carbethoxyl-glycyl-glycine amide and carbethoxyl-glycyl-glycyl-leucine ester give the biuret reaction as would be expected from the researches of Schiff in 1900, who found that glycine amide $NH_2 . CH_2 . CO . NH_2$ also gave the reaction.

In the same way alanyl-alanine and alanyl-alanine ester, which yielded carbethoxyl-alanyl-alanine ester when treated with ethylchloro-carbonate, can be obtained from alanine anhydride, and leucyl-leucine from leucine anhydride or leucinimide, which was first obtained in 1849 by Bopp, and regarded as occurring in the protein molecule, by hydrolysis with hydrobromic acid.

The condensation together of other amino acids in this way by heating their esters is accompanied by difficulties. The diketopiperazine ring is not easily split open by means of acid, and although Fischer, in 1905, discovered that the diketopiperazine could be converted into the dipeptide somewhat easily by treatment with the equimolecular quantity of caustic soda in 10-15 minutes at the ordinary temperature, whereby glycyl-glycine and alanyl-alanine could be easily prepared, yet, in other cases, such as that of leucine anhydride, the anhydride was very resistant to alkali. It appears that the stability of the diketopiperazine ring is connected with the nature of the alkyl groups attached to it, and that there is here another instance of steric hindrance.

The dipeptides of the oxy- and diamino acids have so far only been prepared by this method by Fischer and Suzuki; here, the methyl esters of the amino acids were found to be most easily converted into the anhydrides, and hydrolysis by alkali proceeded readily. The compounds thus obtained can be seen from the accompanying list.

Several of the dipeptides are most readily prepared in this way, and they have been employed in the synthesis of more complex polypeptides. The method, however, does not lend itself to the preparation of higher polypeptides, but it will be observed that pentaglycyl-glycine and another compound, probably octaglycine anhydride, have been prepared by heating the methyl ester of diglycyl-glycine. The various compounds isolated by Curtius and his pupils, such as glycine anhydride and the biuret base, have been obtained from glycine ester.

It must be noted that anhydrides are also formed when the dipeptides, prepared by the other methods, are heated to their melting-points, *e.g.*, leucyl-proline anhydride from leucyl-proline.

Mixed anhydrides, as for example glycyl-alanine anhydride, cannot be obtained by heating a mixture of the esters, where a complex mixture would result, but they are easily prepared by the action of ammonia upon the esters of the dipeptides. These compounds, of which several have now been prepared, are of great importance as they serve for the isolation of dipeptides from a mixture of polypeptides and amino acids (see polypeptides isolated from proteins). On hydrolysis they yield a mixture of the two dipeptides, composed of the amino acids of which they are built up. Thus glycyl-l-tyrosine anhydride yielded glycyl-l-tyrosine and l-tyrosyl-glycine. The latter compound is the first example of a polypeptide containing the tyrosine radical as the acyl group; in all the other polypeptides in which tyrosine is present it stands at the end of the chain.

POLYPEPTIDES SYNTHESISED BY METHOD I.

Simple Polypeptides.

Glycine ester → glycine anhydride → glycyl-glycine.
Alanine ester → alanine anhydride → alanyl-alanine.
Leucine ester → leucine anhydride → leucyl-leucine.
Diaminopropionic acid ester → diaminopropionic acid anhydride.
Histidine ester → histidine anhydride → histidyl-histidine.
Lysine ester → lysine anhydride → lysyl-lysine.
Arginine ester → arginyl-arginine (?)
Serine ester → serine anhydride → seryl-serine.
Isoserine ester → isoseryl-isoserine.
Tyrosine methyl ester → tyrosine anhydride → tyrosyl-tyrosine.
Aspartic acid methyl ester → aspartic acid anhydride → diketopiperazine diacetic diamide by action of ammonia.
2-5-diketopiperazine, 3-6-diacetic acid

Diglycyl-glycine methyl ester → pentaglycyl-glycine ester → pentaglycyl-glycine.
→ octaglycine anhydride (?)

l-alanyl-glycyl-glycine methyl ester → l-alanyl-diglycyl-l-alanyl-glycyl-glycine.

Leucyl-alanine anhydride, Leucyl-proline anhydride } from dipeptide.

Mixed Polypeptides.

Chloracetyl alanine ester → Glycyl-alanine anhydride → glycyl-alanine. / alanyl-glycine.

Chloracetyl tyrosine ester → glycyl-l-tyrosine anhydride → glycyl-l-tyrosine. / l-tyrosyl-glycine.

Glycyl-l-phenylalanine ester, l-phenylalanyl-glycine ester → glycyl-l-phenylalanine anhydride

Leucyl-glycine ester → leucyl-glycine anhydride → leucyl-glycine. / glycyl-leucine.

Glycyl-aspartic acid ester → glycyl-aspartic acid anhydride

leucyl-alanine anhydride → leucyl-alanine. / alanyl-leucine.

Phenylalanyl-glycine ester → Phenylalanyl-glycine anhydride.
Valyl-glycine ester → Valyl-glycine anhydride.
Valyl-alanine ester → Valyl-alanine anhydride.

II. Synthesis of Polypeptides by Means of the Halogen Acyl Compounds.

E. Fischer and E. Otto first described this method of synthesising polypeptides in 1903. Just as an ordinary acyl radical can be combined with an amino acid, *e.g.*, in the preparation of benzoylalanine, so also can a halogen substituted acyl radical be combined with an amino acid. The subsequent action of ammonia upon this compound replaces the halogen atom by the amino group and a dipeptide results, thus :—

Chloracetylchloride and alanine yield chloracetylalanine,

$$Cl.CH_2.COCl + NH_2.CH(CH_3)COOH = Cl.CH_2.CO-NH.CH(CH_3)COOH + HCl,$$

from which, by the action of ammonia, glycyl-alanine is obtained :—

$$Cl.CH_2.CO-NH.CH(CH_3).COOH + 2NH_3 = NH_2.CH_2.CO-NH.CH(CH_3)COOH + NH_4Cl.$$

In practice this reaction can be carried out in two ways :—

1. By the action of the halogen acylchloride upon the alkaline solution of the amino acid. This reaction proceeds well with the higher acylchlorides which are not rapidly acted upon by water, but with the lower acylchlorides it must be carried out at a very low temperature, and the yields even then are in many cases very poor.

2. By the action of the halogen acylchloride upon the ester of the amino acid in anhydrous solvents, such as ether, chloroform, petroleum ether. In this reaction two molecules of amino acid ester are required for one molecule of halogen acylchloride, since half the ester is removed from the reaction as ester hydrochloride. In order to prevent this, the reaction may be carried out in the presence of alkali or alkali carbonate. Subsequent saponification of the ester follows this operation, and loss results by the action of alkali on the halogen acyl radical. This method is only used when the reaction gives bad yields in aqueous solution.

Several halogen acylchlorides are necessary for introducing the various amino acid radicals. These are :—

Chloracetyl-chloride for the introduction of the glycyl radical.
α-Bromopropionyl-chloride for the introduction of the alanyl radical.
l-α-Bromopropionyl-chloride for the introduction of the d-alanyl radical.
α-Bromobutyryl-chloride for the introduction of the α-aminobutyryl radical.
α-Bromisocapronyl-chloride for the introduction of the leucyl radical.
α-Bromophenylacetyl-chloride for the introduction of the phenylglycyl radical.
α-Bromo-hydrocinnamyl-chloride for the introduction of the phenylalanyl radical.
Phenyl-bromopropionyl-chloride for the introduction of the phenylalanyl radical.
α-δ-Dibromovaleryl-chloride for the introduction of the prolyl radical.
Fumaryl-chloride for the introduction of the asparagyl radical.

The introduction of the prolyl group into an amino acid by means

of α-δ-dibromovaleric acid chloride reminds us of the synthesis of proline, where when the compound is treated with ammonia in order to exchange the Br atoms for NH_2, ammonia is lost and ring formation occurs. Prolyl-alanine is prepared as follows:—

$$CH_2Br.CH_2.CH_2.CHBr.COCl + NH_2.CH(CH_3).COOH$$
$$= HCl + CH_2Br.CH_2.CH_2.CHBr.CO—NH.CH(CH_3)COOH$$
$$CH_2Br.CH_2.CH_2.CHBr.CO—NH.CH(CH_3).COOH + 3NH_3$$
$$= 2NH_4Br + CH_2.CH_2.CH_2.CH.CO—NH.CH(CH_3)COOH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \backslash NH /$$

In order to introduce the asparagyl group into an amino acid, chlorosuccinyl chloride, the corresponding halogen-acyl chloride, cannot be employed, since on treatment with ammonia it yields fumaryl derivatives. These, however, when heated with strong ammonia again take up ammonia forming the asparagyl compound, and hence can be employed for this purpose.

These radicals can be introduced into all the simple mono-amino acids, such as alanine, leucine, tyrosine, etc.; also into cystine and the dicarboxylic acids when the compounds such as dialanyl-cystine and asparagyl-dialanine are formed. They can also be introduced into the molecule of a di, tripeptide, etc., as can be seen from the appended list of polypeptides synthesised by this method, which, however, only allows of the chain of amino acids being lengthened on one side, namely, at the amino group end.

The majority of the polypeptides synthesised by this method are optically inactive, but the optically active compounds can also be prepared by employing the optically active halogen-acyl chloride. As previously described under the optically active amino acids, these compounds undergo the Walden inversion; the method therefore allows of the whole of an inactive amino acid being employed for the synthesis of an optically active polypeptide; thus dl-leucine after separation into d-leucine and l-leucine can be converted into l-leucyl-l-leucine by preparing the d-bromisocapronyl chloride from the d-leucine and combining it with l-leucine; treatment with ammonia gives l-leucyl-l-leucine as the compound undergoes the Walden inversion. The four isomers

$$\left.\begin{matrix}\text{d-leucyl-l-leucine}\\ \text{l-leucyl-d-leucine}\end{matrix}\right\} \text{A} \qquad \left.\begin{matrix}\text{d-leucyl-d-leucine}\\ \text{l-leucyl-l-leucine}\end{matrix}\right\} \text{B}$$

can be thus prepared. The A compound is the former inactive leucyl-leucine. Also l-phenylalanyl-glycine was obtained from d-phenyl-α-bromopropionylchloride and glycine, and glycyl-l-phenylalanine from chloracetylchloride and l-phenylalanine, the dl-phenylalanine used having been separated into its optical isomers by means of its formyl compound.

POLYPEPTIDES SYNTHESISED BY METHOD II.

Dipeptides.

Inactive.	Optically Active.
Glycyl-alanine.	Glycyl-l-tyrosine.
Glycyl-phenylalanine.	Glycyl-d-alanine.
Glycyl-leucine.	Glycyl-d-tryptophane.
Glycyl-asparagine.	Glycyl-l-phenylalanine.
	Glycyl-3, 5-diiodo-l-tyrosine.
Alanyl-glycine.	dl-Alanyl-d-alanine.
Alanyl-alanine.	l-Alanyl-glycine.
Alanyl-leucine A.	d-Alanyl-d-alanine.
Alanyl-leucine B.	d-Alanyl-l-leucine.
Alanyl-phenylalanine.	d-Alanyl-d-tryptophane.
α-Aminobutyryl-glycine.	
α-Aminobutyryl-aminobutyric acid A.	
α-Aminobutyryl-aminobutyric acid B.	
Valyl-glycine.	
Valyl-alanine.	
Leucyl-glycine.	l-Leucyl-l-tyrosine.
Leucyl-alanine.	l-Leucyl-glycine.
Leucyl-leucine A.	l-Leucyl-d-alanine.
Leucyl-leucine B.	l-Leucyl-l-leucine.
Leucyl-phenylalanine α.	d-Leucyl-l-leucine.
Leucyl-phenylalanine β.	d-Leucyl-d-leucine.
Leucyl-isoserine A.	l-Leucyl-d-leucine.
Leucyl-isoserine B.	d-Leucyl-l-asparagine.
Leucyl-asparagine.	l-Leucyl-l-asparagine.
Leucyl-aspartic acid.	l-Leucyl-d-tryptophane.
Leucyl-proline.	l-Leucyl-d-glutamic acid.
Phenylglycyl-glycine.	
Phenylglycyl-alanine A.	
Phenylglycyl-alanine B.	
Phenylglycyl-asparagine.	
Phenylalanyl-glycine.	l-Phenylalanyl-glycine.
Phenylalanyl-alanine.	
Phenylalanyl-leucine.	
Phenylalanyl-phenylalanine.	
Asparagyl-mono-glycine.	
Prolyl-alanine.	

Tripeptides.

Diglycyl-glycine (chloracetylchloride + glycyl-glycine ester).
Alanyl-glycyl-glycine (α-bromopropionylbromide + glycyl-glycine ester).
Leucyl-glycyl-glycine (α-bromisocapronylchloride + glycyl-glycine ester or + glycine anhydride + NaOH).
Phenylalanyl-glycyl-glycine (phenyl-α-bromopropionylchloride + glycyl-glycine).
Leucyl-α-leucyl-phenylalanine (α-bromisocapronylchloride + α-leucyl-phenylalanine).
Leucyl-glycyl-phenylalanine (α-bromisocapronylchloride + glycyl-phenylalanine).
Diglycyl-phenylalanine (chloracetylchloride + glycyl-phenylalanine).
Diglycyl-cystine (chloracetylchloride + cystine).

Dialanyl-cystine (α-bromopropionylbromide + cystine).
Dileucyl-cystine (α-bromisocapronylchloride + cystine).
Aspargyl-dialanine (fumarylchloride + alanine ester).
Leucyl-alanyl-glycine A
Leucyl-alanyl-glycine B } (α-bromisocapronylchloride + alanyl-glycine).
Alanyl-leucyl-glycine (α-bromopropionylbromide + leucyl-glycine).
Glycyl-leucyl-alanine (chloracetylchloride + leucyl-alanine).
Leucyl-alanyl-alanine A
Leucyl-alanyl-alanine B } (α-bromisocapronylchloride + alanyl-alanine).
Dialanyl-alanine (α-bromopropionylbromide + alanyl-alanine).
l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine ester).
l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine).
d-Alanyl-glycyl-l-tyrosine (d-α-bromopropionylchloride + glycyl-l-tyrosine).
l-Leucyl-glycyl-d-tryptophane (d-α-bromisocapronylchloride + glycyl-d-tryptophane).

Tetrapeptides.

Triglycyl-glycine (chloracetylchloride + diglycyl-glycine).
Dileucyl-glycyl-glycine (α-bromisocapronylchloride + leucyl-glycyl-glycine).
l-Leucyl-diglycyl-glycine (d-α-bromisocapronylchloride + diglycyl-glycine).

Pentapeptides.

Tetraglycyl-glycine (chloracetylchloride + triglycyl-glycine).

III. Synthesis of Polypeptides by Means of the Acid Chlorides of the Amino Acids and of the Polypeptides.

This, the simplest method of combining together two or more amino acids, is the one, in contradistinction to the previous one, by which the chain of amino acids can be lengthened at the carboxyl end of the molecule. It could not be employed at the commencement of Emil Fischer's researches, since the acid chlorides of the amino acids were unknown, and all attempts to prepare them had failed; but it is now of the greatest importance, as it admits of the preparation of any conceivable polypeptide, and it has also given us the knowledge of the most complex compound known by synthesis.

Although the acid chlorides of the amino acids themselves were unknown, it was found by Fischer that their carbethoxyl derivatives, as also those of the dipeptides which had been prepared, could be converted into their acid chlorides by the action of thionyl chloride, and that these compounds could be combined with the esters of the amino acids or of polypeptides, thus:—

Carbethoxyl-glycyl chloride and glycine ester yielded carbethoxyl-glycyl-glycine ester.

Carbethoxyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl diglycyl-glycine ester.

Carbethoxyl-glycyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl-triglycyl-glycine ester.

This last compound on hydrolysis gave the free acid, which contains four glycyl groups, and was the first known representative of the tetrapeptides.

In the same way derivatives of mixed polypeptides could be obtained, *e.g.*, carbethoxyl-glycyl-alanine ester. From it, by the action of ammonia, Fischer and Otto prepared the amide and, by saponification with soda, the free acid, but the preparation of the simple polypeptide could not be effected, since it was impossible to remove the carbethoxyl group without complete destruction of the molecule.

In 1904 Fischer found that the presence of a halogen acyl group in the molecule of an amino acid again allowed of the preparation of the acid chloride, *i.e.*, when the amino group of the amino acid was rendered stable, and that this compound was formed by the action of phosphorus pentachloride in the presence of acetyl chloride. As before, this acid chloride could be combined with the esters of amino acids or of polypeptides, *e.g.*—

Bromisocapronylglycine was converted into its acid chloride and

combined with glycine ester, when it yielded bromisocapronyl-glycyl-glycine ester,

$$C_4H_9 . CHBr . CO — NH . CH_2 . COCl + 2NH_2 . CH_2 . COOC_2H_5 = HCl . NH_2 . CH_2 . COOC_2H_5 + C_4H_9 . CHBr . CO — NH . CH_2 . CO — NH . CH_2 . COOC_2H_5$$

which on subsequent saponification and treatment with ammonia, gave the tripeptide leucyl-glycyl-glycine,

$$C_4H_9 . CH(NH_2) . CO — NH . CH_2 . CO — NH . CH_2 . COOH.$$

If combined with glycyl-glycine ester and treated in the same way the tetrapeptide leucyl-diglycyl-glycine,

$$C_4H_9 . CH(NH_2) . CO — NH . CH_2 . CO — NH . CH_2 . CO — NH . CH_2 . COOH,$$

was obtained.

Not only was it possible to prepare the acid chloride of a halogen acyl derivative of an amino acid, but also that of a di-, tri-, etc., peptide by exactly the same means. Thus, the compound bromisocapronyl-diglycyl-glycyl chloride can be obtained, and by condensing it with the esters of amino acids and of polypeptides Fischer has prepared a hexa-, a hepta-, and a deca- peptide (see tabulation).

These compounds already exhibit the extraordinary possibilities of synthesis by this method. By continuing the process of preparing the acid chloride of a new polypeptide and again combining it with a polypeptide ester, the synthesis of the complex octadecapeptide, composed of fifteen glycine residues and three leucine residues, was effected in 1907. Its preparation is the best illustration of how this method lends itself to the synthesis of the polypeptides.

Bromisocapronyl diglycyl-glycine was converted into its acid chloride and combined with pentaglycyl-glycine. The resulting bromo compound was treated with liquid ammonia and the decapeptide l-leucyl-octaglycyl-glycine,

$$C_4H_9 . CH(NH_2) . CO — (NH . CH_2 . CO)_8 — NH . CH_2 . COOH,$$

was obtained. This gave, on combination with bromisocapronyl diglycyl-glycyl chloride and subsequent treatment with ammonia, the tetradecapeptide, leucyl-triglycyl-leucyl-octaglycyl-glycine,

$$C_4H_9 . CH(NH_2)CO — (NH . CH_2 . CO)_3 — C_4H_9 . CH(NH_2) . CO — (NH . CH_2 . CO)_8 — NH . CH_2 . COOH.$$

A repetition of the process of combining this new compound with bromisocapronyl-diglycyl-glycyl chloride and treating with ammonia yielded the octadecapeptide, leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine,

$$C_4H_9 . CH(NH_2)CO — (NH . CH_2 . CO)_3 — C_4H_9 . CH(NH_2) . CO — (NH . CH_2 . CO)_3 — C_4H_9 . CH(NH_2) . CO — (NH . CH_2 . CO)_8 — NH . CH_2 . COOH.$$

In the preparation of this octadecapeptide complete combination of the polypeptide with the acid chloride was very essential, since if the greater part of the compound be not used up, but remained unchanged, it was precipitated with the bromo compound on acidifying; this was only attained by using a very large excess of the acid chloride. At the same time there was the technical difficulty of frothing; this was overcome by shaking with glass beads in large flasks. Liquid ammonia was also necessary for the conversion of the halogen compound into its amino derivative. Analysis of the polypeptides hardly sufficed for the determination of their synthesis, since the variations in the figures are so small, but a determination of the bromine in the corresponding halogen derivative indicated that the synthesis was being effected in the stages represented.

This octadecapeptide has the highest molecular weight of any compound as yet prepared by synthesis and of which we know the constitution. Its molecular weight is 1213, a figure which far exceeds that of the fats, tristearin having a molecular weight of only 891. If the compound contained other amino acid residues, such as leucine, tyrosine, phenylalanine in the place of the glycine residues, the molecular weight would be increased two to three times.

Such a figure of 3,000-5,000 has been found for the molecular weight of many proteins, and it would appear that they are composed of some twenty amino acids. The higher values of 12,000-15,000 which have been found for the molecular weight of other proteins, are, according to Fischer, very doubtful, since we have no indication of their purity, in spite of the crystallisability of many of them; the admixture of a small quantity of another protein might easily raise the value to this extent.

Fischer subsequently found that the acid chloride of other acyl derivatives of the amino acids could be prepared by the same process. Thus, by treating finely powdered hippuric acid with phosphorus pentachloride in the presence of acetyl chloride, he obtained hippuryl chloride, a compound which numerous investigators had tried to synthesise, but unsuccessfully. By combining hippuryl chloride with glycine ester, benzoyl-glycyl-glycine was obtained, and this compound, when converted into its acid chloride and combined with glycine, yielded benzoyl-diglycyl-glycine. In this way, by means of the acid chlorides, Fischer has prepared the same compounds which Curtius has prepared by means of hippurazide.

By applying this method of preparing the acid chlorides to the amino acids themselves, Fischer ultimately succeeded in obtaining the

amino acid chlorides, so that he was enabled to combine together any two amino acids in any order, without the necessity of preparing the corresponding halogen derivative. Polypeptides containing the natural optically active amino acids can thus be synthesised with ease, since the natural compound obtained by hydrolysis can be again used directly in the synthesis, and very often it is easier to prepare the natural compound than the synthetical one, which also requires separation into its stereoisomers.

The synthesis of polypeptides composed of different amino acids is most easily effected by this method. Those containing tyrosine are of particular interest, since the first natural tetrapeptide was isolated from silk in 1907, and was composed of glycine, alanine and tyrosine. Twelve isomers are possible for a tetrapeptide of this composition, but if the results of partial hydrolysis and subsequent anhydride formation be taken into account, this number is reduced to eight. Of these, glycyl-d-alanyl-glycyl-l-tyrosine was synthesised in 1908 by combining chloracetyl-d-alanyl-glycylchloride with l-tyrosine ester:—

$$Cl.CH_2.CO{-}NH.CH(CH_3).CO{-}NH.CH_2.COCl + 2NH_2.CH(C_6H_4OH).COOCH_3 = HCl.NH_2.CH(C_6H_4OH).COOCH_3 + Cl.CH_2.CO{-}NH.CH(CH_3).CO{-}NH.CH_2.CO{-}NH.CH(C_6H_4OH).COOCH_3$$

saponifying the resulting chloro compound with caustic soda and treating with aqueous ammonia:—

$$Cl.CH_2.CO{-}NH.CH(CH_3).CO{-}NH.CH_2.CO{-}NH.CH(C_6H_4OH).COOCH_3 + H_2O + 2NH_3 = CH_3OH + NH_4Cl + NH_2.CH_2.CO{-}NH.CH(CH_3).CO{-}NH.CH_2.CO{-}NH.CH(C_6H_4OH).COOH$$

This product, though it had many points of resemblance, such as precipitation by phosphotungstic acid, tannic acid, hydrolysis by trypsin, with the natural tetrapeptide, was not, however, identical with it; it differed mainly in its behaviour to ammonium sulphate, by which it was only salted out with great difficulty.

An attempt was made at the same time to prepare the isomeric d-alanyl-glycyl-glycyl-l-tyrosine; it failed on account of the difficulty of preparing pure *α*-bromopropionyl-glycyl-glycyl-chloride, but there seems no reason to suppose that Fischer will not overcome this small difficulty in preparing a desired compound, when he has overcome such vast difficulties already in connection with the synthesis of the polypeptides.

The accompanying table gives a list of the compounds prepared by this method.

POLYPEPTIDES SYNTHESISED BY METHOD III.

Dipeptides.

Benzoylglycyl-glycine (hippuryl chloride + glycine ester).
d-Alanyl-glycine (d-alanyl chloride + glycine ester).
d-Alanyl-d-alanine (d-alanyl chloride + d-alanine ester).
dl-Valyl-glycine.
Valyl-alanine A.
l-Leucyl-glycine (l-leucyl chloride + glycine ester).
l-Leucyl-d-alanine (l-leucyl chloride + d-alanine ester).
l-Leucyl-l-leucine (l-leucyl chloride + l-leucine ester).
d-Tryptophyl-glycine (d-tryptophyl chloride + glycine ester).

Tripeptides.

Benzoyl-diglycyl-glycine (benzoyl-glycyl-glycyl chloride + glycine ester).
Leucyl-glycyl-glycine (leucyl-glycyl chloride + glycine ester).
Leucyl-glycyl-leucine (leucyl-glycyl chloride + leucine ester).
Glycyl-l-asparagyl-l-leucine (chloracetyl-l-asparagyl chloride + l-leucine ester).

Tetrapeptides.

Leucyl-diglycyl-glycine (bromisocapronyl-glycyl-glycyl chloride + glycine ester).
,, ,, ,, (bromisocapronyl-glycyl chloride + glycyl-glycine ester).
,, ,, ,, (leucyl-diglycyl chloride + glycine ester).
Glycyl-d-alanyl-glycyl-l-tyrosine (chloracetyl-d-alanyl-glycyl chloride + l-tyrosine ester).

Pentapeptide.

Leucyl-triglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + glycine ester).

Hexapeptide.

Leucyl-tetraglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + glycyl-glycine).

Heptapeptide.

Leucyl-pentaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + diglycyl-glycine).

Octapeptide.

Leucyl-hexaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + triglycyl-glycine).

Decapeptide.

Leucyl-octaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + pentaglycyl-glycine).

Dodecapeptide.

Leucyl-decaglycyl-glycine (bromisocapronyl-tetraglycyl-glycyl chloride + pentaglycyl-glycine).

Tetradecapeptide.

Leucyl-triglycyl-leucyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + l-leucyl-octaglycyl-glycine).

Octadecapeptide.

Leucyl-triglycyl-leucyl-triglycyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + leucyl-triglycyl-leucyl-octaglycyl-glycine).

The Structure of the Polypeptides and Diketopiperazines.

From the methods by which the polypeptides are obtained by synthesis it can only be concluded that their constituent amino acids are combined together in the form of acid amides; this method of combination also occurs in the case of the oxyamino acids, *e.g.*, leucyl-isoserine, where the ester method of combination was excluded by special investigations. The question of their structure, however, still remains very complex, if the controversy concerning the structure of the amides and amino acids, which has not yet been settled, be taken into account. There is the possibility of lactam and lactim forms and of the free amino acid and intramolecular salt; these are illustrated by the four formulæ for glycyl-glycine:—

$NH_2 . CH_2 . CO—NH . CH_2 . COOH$
$NH_3 . CH_2 . CO—NH . CH_2 . COO$

$NH_2 . CH_2 . C(OH)=N . CH_2 . COOH$
$NH_3 . CH_2 . C(OH)=N . CH_2 . COO$

For the sake of simplicity and since his observations have as yet led to no choice between the above formulæ, Fischer has adopted the first formula, but in certain of the polypeptides the observations suggest different structures, which will increase with the investigations upon the more complex polypeptides, thus leucyl-diglycyl-glycine in its amorphous state is easily soluble in alcohol; if the alcohol solution be warmed on the water bath, the crystalline tetrapeptide commences to separate out and in this state it is insoluble in alcohol.

The carbethoxyl derivatives show another kind of isomerism; carbethoxyl glycyl-glycine ester, when saponified by alkali, yielded glycyl-glycyl carboxylic acid $HOOC . NH . CH_2 . CO . NH . CH_2 . COOH$; this compound on esterification by alcoholic hydrochloric acid yielded a neutral ester, and this compound was isomeric with the former one. The experiments of Leuchs (p. 18) have shown their exact nature, and they are designated as the *a*- and *β*-compounds. A similar isomerism occurs with carbethoxyl diglycyl-glycine ester and with the corresponding double amides.

Polypeptides, which contain amino dicarboxylic acids or diamino acids can also exist in isomeric forms; asparagyl-monoglycine can exist in the two forms,

$CO—NH . CH_2 . COOH$
$CH . NH_2$
$CH_2 . COOH$

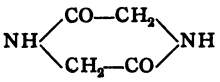
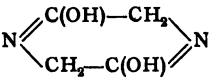
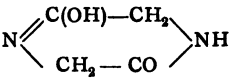
and

$COOH$
$CH . NH_2$
$CH_2 . CO—NH . CH_2 . COOH$

as also the dipeptide of diaminopropionic acid:—

$$NH_2.CH_2.CH(NH_2).CO-NH.CH_2.CH(NH_2).COOH$$
$$NH_2.CH_2.CH(NH_2).CO-NH.CH.COOH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad CH_2.NH_2$$

The diketopiperazines which are so closely related to the dipeptides can also occur in a keto- or -enol form; the possibilities are—

The existence of the -enol form was emphasised by the fact that in the hydrolysis of alanine anhydride by alkali, a transient formation of an alkali compound was observed.

The Configuration of the Polypeptides.

Excepting glycine all the amino acids employed in the previous syntheses contain an asymmetric carbon atom. According to the law of van't Hoff, the polypeptides will therefore occur in 2^n forms. Thus, a dipeptide,

$$NH_2.CHR.CO-NH.CHR.COOH,$$

containing two asymmetric carbon atoms will be capable of existence in the four active forms

dd′ ll′ dl′ ld′

of which the two first and the two last together will form a racemic compound.

A tripeptide can exist in 2^8 forms, *i.e.*, eight, a tetrapeptide in 2^4 or sixteen forms, etc.

The two inactive forms of a dipeptide are obtained when the two optically inactive compounds are coupled together by synthesis, and they appear first in the form of the corresponding halogen derivative,

$$Br.CHR.CO-NH.CHR.COOH.$$

A separation of the two racemic forms has been effected in certain cases at this stage, *e.g.*, leucyl-phenylalanine, but in the majority of cases only one product has been isolated. The formation of only one product in the reaction may be due either to the influence of stereoisomerism upon the combination of the compounds, which is especially noticeable when enzymes are concerned, or it may be due to a difference in the rate of combination of the two compounds, which was first observed by Markwald and Mackenzie with simple compounds. The latter explanation is the more probable, since when both compounds have been isolated, their amounts have been very different. There still remains the possibility that the single substance isolated is still a

mixture of the two compounds, for the separation of mixed crystals of similar compounds is of the greatest difficulty.

Concerning the nomenclature of the two compounds where they have been isolated, the more insoluble is called the A compound and the more soluble the B compound. It has been possible to determine by the action of trypsin, which only hydrolyses the compound containing the naturally occurring amino acids, what combinations are present in them; thus, as alanyl-leucine A was hydrolysed by trypsin it must contain d-alanyl-l-leucine, and the two compounds must therefore be

d-alanyl-l-leucine
l-alanyl-d-leucine } A

d-alanyl-d-leucine
l-alanyl-l-leucine } B

This has been proved by the later work upon the optically active polypeptides composed of these amino acids.

One product only can result when the two components consisting of the pure optically active amino acids are combined together, *e.g.*—

d-alanyl-d-alanine

from d-alanylchloride and d-alanine ester.

Two products again result when one of the components is optically active and the other racemic. The various combinations of optically active tyrosine and aspartic acid with racemic leucine, alanine, etc., come into this category; they are designated as, *e.g.*—

dl-alanyl-l-tyrosine
glycyl-dl-leucine

These compounds are not optical antipodes, and can therefore be separated by simple crystallisation. In the case of the leucyl-asparagines,

d-leucyl-l-asparagine
l-leucyl-l-asparagine

this separation has been effected, but in the majority of cases no separation was carried out, since the similarity of the isomers was so great that they formed apparent mixed crystals. Such a condition was termed by Fischer in 1894 "partial racemism". It occurs almost always when a racemic compound in combination with an active residue cannot be separated into its two isomeric forms by simple crystallisation.

Cystine, as its constitution shows,

$$COOH.CH(NH_2).CH_2.S.S.CH_2.CH(NH_2).COOH,$$

resembles the tartaric acids in its stereochemistry; it is composed of two exactly similar halves, and it matters very little with which amino group combination is effected. But if it be combined with two mole-

cules of a racemic acid chloride, *e.g.*, *a*-bromopropionyl chloride, three isomeric optically active compounds can result, namely:—

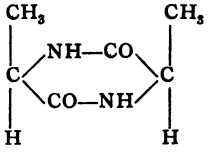
d-bromopropionyl-d-bromopropionyl-cystine
l-bromopropionyl-l-bromopropionyl-cystine
d-bromopropionyl-l-bromopropionyl-cystine

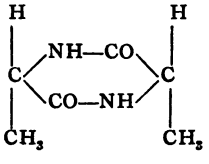
A yield of 71 per cent. of dibromopropionyl-cystine was obtained by Fischer and Suzuki and was apparently a definite substance. It was therefore regarded as the dl-compound, since its formation is independent of the formation of the dd- or ll-compounds which most probably would result in equal amounts.

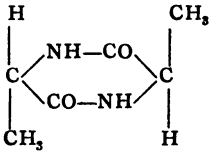
The Configuration of the 2, 5- Diketopiperazines.

Diketopiperazines composed of two molecules of the same amino acids, *i.e.*, containing the same substituting group, can, according to theory, exist in four forms, which are comparable to those of the tartaric acids, namely:—

1. The active dextro form, containing the two dextro rotating molecules.
2. The active laevo form, containing the two laevo rotating molecules.
3. The inactive form, a mixture of 1 and 2.
4. The inactive form, containing a dextro and a laevo rotating molecule, as can be seen from the structural formulæ for the anhydrides of alanine:—







The substituting groups in the optically active forms are thus in the *cis*-position, in the inactive meso form in the *trans*-position.

The proof of the existence of these various forms was commenced in 1906 by Fischer and Raske in the case of the alanine anhydrides. They prepared the inactive trans-anhydride

1. by the action of ammonia upon l-alanyl-d-alanine ester;
2. by the action of ammonia upon d-alanyl-l-alanine ester.

The active d-alanine anhydride had been previously synthesised in a similar way from d-alanyl-d-alanine ester, and an inactive anhydride

had been obtained by heating inactive alanine ester; this probably represents the inactive mixture of the dextro and laevo anhydrides; the other compound l-alanine anhydride has not yet been prepared.

The several forms of the diketopiperazines were synthesised in 1907 by Fischer and Koelker, who prepared

1. d-leucine anhydride from d-leucyl-d-leucine ester and ammonia;
2. l-leucine anhydride from l-leucyl-l-leucine ester and ammonia;
3. trans-leucine anhydride from d-leucyl-l-leucine ester and ammonia and from l-leucyl-d-leucine ester and ammonia.

Hydrolysis by alkali of these anhydrides should give the corresponding dipeptide, but in the case of the aminobutyryl-aminobutyric acid anhydrides Fischer and Raske have found that a steric rearrangement occurred; the dipeptide A was obtained both from the anhydride A and B; these had been prepared by the action of ammonia on the respective inactive esters, so that in this manner the dipeptide B can be converted into the dipeptide A.

The number of isomers of diketopiperazines containing two molecules of different amino acids is greater than when the two amino acids in the molecule are the same. It can be calculated from the number of asymmetric carbon atoms in the molecule just as in the open chain compounds; thus alanyl-leucine anhydride can exist in four optically active forms and two racemic forms:—

1. d-alanyl-d-leucine anhydride;
2. d-alanyl-l-leucine „
3. l-alanyl-d-leucine „
4. l-alanyl-l-leucine „
5. a mixture of 1 and 4;
6. a mixture of 2 and 3.

The same diketopiperazines are obtainable either from the dipeptides alanyl-leucine or from the dipeptides leucyl-alanine, so that in fact the number of isomers of a diketopiperazine composed of two different amino acids is less than those of the isomers of the dipeptide composed of the same two amino acids.

At present only a racemic form prepared from leucyl-alanine by fusion has been obtained; its nature has not yet been determined.

The Properties of the Polypeptides.

The physical properties of the various polypeptides show generally much resemblance to one another, although many differences have been observed.

The majority are easily soluble in water; the exceptions amongst the dipeptides are, *e.g.*, dl-leucyl-glycine, leucyl-alanine, and leucyl-leucine; also phenylalanyl-glycine, phenylalanyl-phenylalanine, and some others; amongst the tripeptides, leucyl-alanyl-alanine A, phenyl-alanyl-glycyl-glycine, leucyl-glycyl-phenylalanine; amongst the tetra-peptides, dileucyl-glycyl-glycine. In contradistinction to the other polypeptides made up entirely of glycine units, the pentapeptide and the hexapeptide are soluble with difficulty even in hot water.

Of the complex polypeptides, the octapeptide, l-leucyl-hexaglycyl-glycine, is the most soluble in water, and the decapeptide, l-leucyl-octaglycyl-glycine, the least soluble; the solubility increases again in the case of the tetradeca- and octadecapeptides; their warm clear aqueous solutions become opalescent on cooling.

In general, the solubility in water of mixed polypeptides is greater than the solubility of the polypeptides made up of a single amino acid; the ready solubility of the dipeptides glycyl-l-tyrosine, leucyl-tyrosine, which contain the amino acids soluble with difficulty in water, should also be noted.

Most of the polypeptides are insoluble in alcohol; leucyl-proline is, however, an exception, for it dissolves both in alcohol and in acetic ester somewhat easily.

Those polypeptides which are soluble with difficulty in water, are dissolved easily by mineral acids and alkalies with the formation of salts; they are less soluble in acetic acid. In many cases they may be dissolved in alcohol if a few drops of ammonia be added; they separate out on boiling off the ammonia.

Certain polypeptides, for instance, leucyl-diglycyl-glycine, in the amorphous state are soluble in alcohol, but they are changed on warming into their insoluble crystalline form.

Most of the polypeptides melt above 200° C. and at the same time undergo decomposition. The dipeptides when fused are converted into their diketopiperazines. Certain of the glycine polypeptides are decomposed without melting. Leucyl-proline again, as in many other of its properties, is an exception, as it melts at 116-119° C.

The taste of the polypeptides is not sweet, like that of the amino acids, but slightly bitter; some of the isomeric polypeptides possess

distinct differences in their taste; thus leucyl-alanine is tasteless, but the two alanyl-leucines have a bitter taste. The presence of *a*-amino acids amongst polypeptides may even be recognised by their sweet taste, and the resemblance of the polypeptides in their bitter taste to the natural peptones is very remarkable.

The optically active polypeptides have generally a very high specific rotation in comparison with the amino acids; but the rotation is very changeable just as in other classes of compounds. Multirotation has not yet been observed. This property has proved very valuable in the study of the hydrolysis of the polypeptides by the action of enzymes.

The chemical properties of the polypeptides depend greatly on their complexity. Like amino acids, all the ordinary polypeptides, when their solutions are boiled with precipitated copper oxide, give blue, sometimes blue-violet solutions, and in this way differ from the diketo-piperazines, whose solutions remain colourless, *i.e.*, they do not give copper salts. Leucyl-proline forms again an exception.

The high molecular polypeptides, such as the octa-, the deca- up to the tetradeca-peptides, give salts with mineral acids which are soluble with difficulty, but the lower ones give soluble salts as before mentioned.

The simple polypeptides, like the *a*-amino acids, give no precipitate with phosphotungstic acid, but this condition depends on the length of the polypeptide chain. Many tripeptides, such as leucyl-glycyl-glycine, give a precipitate with phosphotungstic acid in the presence of sulphuric acid if their solution be not too dilute, and this occurs with almost all the tetrapeptides. The derivatives of the diamino acids behave as expected in giving a precipitate with phosphotungstic acid.

The octa-, deca-, etc., peptides are immediately precipitated by phosphotungstic acid; they are also thrown down by tannic acid and by concentrated ammonium sulphate solutions. They resemble, in fact, many natural proteins and would have been regarded as such if they had been found in nature. They lack only the colour reactions due to the absence of tyrosine, tryptophane, etc., in their molecules.

The biuret reaction is positive with the greater number of the polypeptides excluding the dipeptides. In the case of the glycine compounds it occurs first with the tetrapeptide, but it occurs with other tripeptides. It is distinctly intenser as the length of the polypeptide chain increases, and the colour is also more intense when the carboxyl group is esterified; this is especially noticeable in the case of triglycyl-glycine and its ester, the so-called biuret base. The same occurs when

the carboxyl group is converted into the acid amide group; here one of the conditions necessary, as shown by Schiff, is added.

The polypeptides yield the same derivatives as the amino acids; the carboxyl group can be converted into the acid chloride and a halogen acyl group can be attached to the amino group. Further, the benzoyl, the carbethoxyl and the naphthalene-sulpho compounds can be easily obtained; the derivatives with the last mentioned are soluble with difficulty and may be made use of for characterising the polypeptides. The combination with phenylisocyanate also takes place readily, but these compounds are not so important as those of the amino acids for purposes of characterisation.

On the other hand, the esters of the polypeptides are of the greatest importance and they are prepared by the action of alcoholic hydrochloric acid. Hydrolysis of the polypeptide does not occur if prolonged heating be avoided, nor does hydrolysis occur when the esters are saponified by dilute cold caustic alkali. The esters have served in particular for the further synthesis of polypeptides and for the isolation of dipeptides from mixtures; on treatment with alcoholic ammonia, the dipeptide esters are converted into their diketopiperazines. They are not soluble in petroleum ether and they are soluble with difficulty in ether, and they thus differ from amino acid esters. Chloroform dissolves them, and in this solvent their combination with acid chlorides has been generally effected.

The polypeptides are attacked by nitrous acid with evolution of nitrogen, but the amino and imino groups show no great difference in their behaviour as might have been expected, and consequently a differentiation of the amino and imino groups in the protein molecule with nitrous acid seems impossible. They are not acted upon by potassium permanganate in the cold, but on boiling they are oxidised as was shown by Pollak in the case of glycyl-glycine.

The synthetical polypeptides are completely hydrolysed by boiling with concentrated hydrochloric acid for five hours; 10 per cent. hydrochloric acid at 100° C. hydrolyses them very slowly, and normal alkali has only a very slight action. Their hydrolysis by enzymes, especially by trypsin, is of such importance that a special section is required for the description of these results.

The Action of Enzymes upon the Polypeptides.

I. The Action of Trypsin.

One of the best proofs that the protein molecule is built up of amino acids coupled together by the methods devised by E. Fischer is given by the action of the various enzymes upon the synthetical polypeptides.

In 1903, soon after the synthesis of a few of the simple dipeptides and their derivatives had been effected, Fischer and Bergell investigated the action of an extract of pancreas upon them and they found that

glycyl-glycine β-naphthalenesulphoglycyl-d-alanine β-naphthalenesulpho-d-alanyl-glycine Di-β-naphthalenesulphotyrosyl-dl-leucine	were not hydrolysed
β-naphthalenesulphoglycyl-l-tyrosine β-naphthaleneglycyl-dl-leucine Carbethoxyl-glycyl-dl-leucine Glycyl-l-tyrosine Leucyl-alanine Alanyl-leucine Leucyl-leucine	were hydrolysed

from which it was obvious that several factors conditioned the hydrolysis by the enzymes of the pancreas, such as the nature of the dipeptide and its configuration: *e.g.*, the racemic compounds were hydrolysed asymmetrically, the natural component, such as l-leucine being split off from carbethoxyl-glycyl-dl-leucine, the remainder not being acted upon. The results coincide with the facts known with regard to the rapid separation of leucine and tyrosine from proteins by the action of trypsin; the other amino acids, such as glycine and alanine, are not obtained during the early stages of digestion.

Fischer and Abderhalden, in 1905, extended these observations by investigating the effect of pancreatic juice prepared by Pawlow from a pancreatic fistula and activated by enterokinase from duodenal juice, *i.e.*, by the action of pure trypsin upon a larger number of polypeptides, and they were able to divide the polypeptides into two distinct classes:—

Those Hydrolysed.	Those not Hydrolysed.
* Alanyl-glycine.	Glycyl-alanine.
* Alanyl-alanine.	Glycyl-glycine.
* Alanyl-leucine A.	Alanyl-leucine B.
* Leucyl-isoserine A.	Leucyl-alanine.
Glycyl-l-tyrosine.	Leucyl-glycine.
Leucyl-l-tyrosine.	Leucyl-leucine.
* Alanyl-glycyl-glycine.	Aminobutyryl-glycine.
* Leucyl-glycyl-glycine.	Aminobutyryl-aminobutyric acid A.

* These are racemic compounds.

Those Hydrolysed.	Those not Hydrolysed.
* Glycyl-leucyl-alanine.	Aminobutyryl-aminobutyric acid B.
* Alanyl-leucyl-glycine.	Valyl-glycine.
Dialanyl-cystine.	Glycyl-phenylalanine.
Dileucyl-cystine.	Leucyl-proline.
Tetraglycyl-glycine.	Diglycyl-glycine.
Triglycyl-glycine ester (Curtius' biuret base).	Triglycyl-glycine.
	Dileucyl-glycyl-glycine.

To which were added in 1907 the following optically active dipeptides:—

d-alanyl-d-alanine.	d-alanyl-l-alanine.
d-alanyl-l-leucine.	l-alanyl-d-alanine.
l-leucyl-l-leucine.	l-leucyl-glycine.
l-leucyl-d-glutamic acid.	l-leucyl-d-leucine.
	d-leucyl-l-leucine.

The hydrolysis of these compounds by the enzyme was determined by the isolation of the individual substances. The isolation of the amino acids soluble with difficulty in water, namely, tyrosine and cystine, presented no great difficulty, since those compounds crystallised out during the process of hydrolysis, but in the other cases the amino acids required separation from unchanged dipeptide. The ester method here again proved its usefulness; the esters of the simple monoamino acids are easily volatile in vacuo and can be characterised by the methods previously described; those of the dipeptides are not volatile and are characterised by conversion into their diketopiperazines or anhydrides by the action of ammonia, which compounds are less soluble than the dipeptides themselves and are thus capable of separation by filtration.

By simply determining the change in rotation, especially when optically active polypeptides were investigated, an indication that hydrolysis was occurring was obtained; as soon as the rotatory power became constant it was assumed that complete hydrolysis had taken place and the solution was examined for the products of hydrolysis.

In all cases the activity of the ferment was first proved, and freedom from bacterial infection was specially guarded against and certified by control experiments.

An examination of the results of hydrolysis by trypsin shows that several factors have an important influence:—

1. The Structure of the Molecule.

Glycyl-alanine, $NH_2.CH_2.CO—NH.CH(CH_3).COOH$, was not hydrolysed, but the isomeric alanyl-glycine, $NH_2.CH(CH_3).CO—NH.CH_2.COOH$, was hydrolysed; again, alanyl-leucine A was hydrolysed, but not leucyl-alanine.

The order in which the amino acids are combined together in the molecule has therefore a very marked effect. Thus, when alanine is

* These are racemic compounds.

the acyl radical, as in alanyl-glycine, alanyl-alanine, alanyl-leucine, hydrolysis occurred, but the reverse happened when leucine, valine or aminobutyric acid were the acyl radicals; in the three cases, leucyl-alanine, leucyl-glycine and leucyl-proline, no hydrolysis took place; here the racemic compound was employed and the resistance might have been due to this factor, but the instance of l-leucyl-glycine appears to confirm the older result.

If tyrosine, isoserine and cystine stood at the end of the chain, trypsin hydrolysed the compound; in the only case examined where tyrosine, combined with β-naphthalene-sulphonic acid, acted as the acyl radical, there was no hydrolysis.

2. The Configuration of the Molecule.

This is of the greatest importance for a polypeptide to be hydrolysed by trypsin, as will be seen from the list of compounds published in 1907. Only those compounds containing the naturally occurring optically active variety of the amino acid are hydrolysed by trypsin.

The compounds marked with an asterisk are racemic and their hydrolysis was effected asymmetrically, only that portion of the molecule containing the natural stereoisomer being attacked. This explained the difference between alanyl-leucine A and alanyl-leucine B; the former probably consisted of d-alanyl-l-leucine + l-alanyl-d-leucine, the first of which contains the natural stereoisomers upon which the hydrolysis depends; the latter would consist of d-alanyl-d-leucine + l-alanyl-l-leucine. The later experiments of 1907 proved this supposition. It is more noticeable in the case of leucyl-leucine, which must have been l-leucyl-d-leucine + d-leucyl-l-leucine since l-leucyl-l-leucine is hydrolysed.

3. The Number of Amino Acids.

Only the various polypeptides containing several glycine radicals come at present under this heading. Several interesting details are at once apparent. Hydrolysis first took place when five glycine radicals, as in tetraglycyl-glycine, are combined together, although it occurred in the ester of triglycyl-glycine, or the biuret base of Curtius, which had been previously examined by Schwarzschild. The length of the glycine chain is therefore of importance, but an alteration in the carboxyl group may have an influence; it is worth noting that Warburg observed that leucine ester was hydrolysed by pancreatic juice, but whether this was due to the trypsin or the lipase in the juice has not yet been determined.

The fact that leucyl-glycyl-glycine was hydrolysed, but not the more complex dileucyl-glycyl-glycine, was probably due to the configuration of the dileucyl group.

4. The Nature of the Enzyme.

In the earlier experiments by Fischer and Bergell it was found that leucyl-alanine was hydrolysed by an extract of pancreas; it was not however hydrolysed by pure pancreatic juice. Such extracts probably contain other enzymes, more especially the autolytic enzyme, which produce the hydrolysis; the later work of Abderhalden and his co-workers upon the action of enzymes from various organs also show that polypeptides not hydrolysed by pure trypsin are attacked by these enzymes (see table, p. 48).

II. The Action of Pepsin.

Amino acids have been described by various authors as occurring together with the proteoses and peptones in a pepsin digest of proteins. One might have expected that pepsin would act upon certain of the synthetical compounds, especially those most easily hydrolysed by trypsin. Pure pepsin, prepared by Pawlow, had however no action upon glycyl-l-tyrosine, leucyl-alanine, leucyl-leucine, dialanyl-cystine, leucyl-glycine, and one must conclude that the chain of amino acids is not yet sufficiently long to allow of attack by pepsin. The amino acids obtained by the digestion of proteins probably arise by the action of other enzymes contained in the enzyme solution employed. Another explanation may be that pepsin acts upon other combinations of amino acids than those which are hydrolysed by trypsin.

III. The Action of Other Enzymes.

Not only are the synthetical polypeptides hydrolysed by the enzyme of the pancreas, but they are also hydrolysed by the enzymes occurring in the animal body.

It was found by Abderhalden and Bergell, in 1903, that glycyl-glycine when subcutaneously introduced into a rabbit was converted into glycine which appeared in the urine, whereas glycine if administered in a similar way was completely burnt up and was not excreted. Abderhalden and Rona subsequently showed that glycyl-l-tyrosine was burnt up in the organism of the dog when injected into the system, and Abderhalden and Samuely observed that this was also the case when cystine, dialanyl-cystine and dileucyl-cystine were subcutaneously introduced. Abderhalden continued these investigations with Teruuchi, with the result that the organism of the dog was found to be able to completely utilise glycyl-glycine, alanyl-alanine and diglycyl-glycine as well as the diketopiperazines, glycine anhydride and alanine anhydride, when they were given by the mouth, just as the animal can utilise proteins or amino acids, the nitrogen contained in these substances being eliminated as urea. To this series of polypeptides capable of being utilised by the

dog Abderhalden and Samuely added dl-leucine and racemic leucyl-leucine, and Abderhalden and Babkin added leucyl-glycine. These results differed from those of Wohlgemuth, who found that the rabbit excreted d-leucine when dl-leucine was given, but Abderhalden and Kautzsch have also found that the rabbit excretes d-leucine when somewhat large doses of dl-leucine are administered, whereas this animal can utilise dl-leucyl-glycine and dl-leucyl-glycyl-glycine. Abderhalden has since found that the rabbit excretes glycine, l-alanine, and d-serine when the diketopiperazines of these amino acids are administered, which points to their hydrolysis into the dipeptide before they are split into the amino acids.

The organs of various animals, such as the dog and rabbit, would thus appear to differ in their power of making use of synthetical polypeptides, but the animal organism as a whole is not so selective as the enzyme of the pancreas which hydrolyses the racemic dipeptide asymmetrically; in the body the racemic compound is completely burnt up, since no dipeptide composed of the optically active variety of the amino acid not occurring in a protein could be isolated. Further, the animal organism is able to utilise polypeptides not hydrolysed by pancreatic juice, so that if such polypeptides are present in the protein, they can still be utilised by the body although unaffected by trypsin.

Although these polypeptides are utilised by the organism of an animal and the nitrogen contained in them excreted as urea, it does not follow from the results of the experiments that these polypeptides are hydrolysed into their constituent amino acids previous to absorption, more especially those which are not acted upon by trypsin.

Great interest therefore is attached to the subsequent work of Abderhalden in conjunction with Teruuchi, Hunter and Rona, which was commenced in 1906 upon the action of extracts and of press-juices of various organs, prepared by Buchner's method of grinding up with sand, mixing with Kieselguhr and pressing out at a pressure of 100-300 atmospheres, whereby the cell enzymes are obtained. A large number of polypeptides were employed for determining the nature of these enzymes in the hope of finding differences between them, and dividing the proteoclastic enzymes into definite groups, especially as we regard enzymes as being extremely selective in their nature, and in the hope also of determining in which organ the hydrolysis of any particular polypeptide took place. An extract of pancreas was previously found to hydrolyse leucyl-alanine, which was not attacked by pure pancreatic juice, but the results show that the enzymes contained in the various organs are not so selective as pure trypsin in their action, and among themselves show decided differences as exemplified in the following tabulation:—

	Glycyl-glycine.	dl-Leucyl-glycine.	Dialanyl-cystine.	Glycyl-dl-alanine.	Glycine anhydride.	Glycyl-l-tyrosine.	Leucyl-leucine.	Leucyl-phenylalanine.	dl-Alanyl-glycyl-glycine.	dl-Leucyl-glycyl-glycine.	dl-Alanyl-glycine.	Glycyl-dl-leucine.	Diglycyl-glycine.	Triglycyl-glycine.
Pyloric Juice	...	...	...	...	...	o	...	...	...	...	...	...	...	...
Duodenal Juice	...	...	...	...	...	o	...	...	...	...	...	...	...	...
Yeast Juice (Endotryptase)	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Papain	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Juice of Nepenthes	...	...	...	...	...	o	...	...	...	...	...	...	...	...
Juice of Germinating Wheat	+	+	+	...	...	...	...	...	...	...	...	...	...	...
Juice of Germinating Lupine	+	+	+	...	...	...	...	...	...	...	...	...	...	...
Juice of Mushroom	...	+	...	...	...	o	...	...	...	...	+	...	+	...
Ox, Liver Extract	+	+	...	...	...	...	...	...	...	...	...	...	...	...
„ „ Juice	...	+	...	+	o	...	o	+	+	+	...	...	...	...
„ Muscle Juice	+ slight	+ slight	...	+ slight	...	...	...	...	...	...	...	...	...	...
Rabbit, Liver Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
„ Kidney Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
„ Muscle Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
Dog, Muscle Juice	+	...	...	...	...	+	...	...	...	...	...	...	...	...
„ Kidney Juice	+	...	...	...	...	...	...	...	...	...	...	...	...	...
„ Liver Juice	+	...	...	...	...	+	...	...	...	...	...	...	...	...
„ Intestinal Juice (Erepsin)	+	...	...	...	...	+	...	...	...	...	...	...	...	...
„ Extract of Intestine	+	...	...	...	...	...	...	...	...	...	...	...	...	...
Pig, Lenses from Eyes	...	...	...	o	...	+	...	...	...	...	+	...	+	...
Calf, Brain	...	...	...	o	...	o	...	...	...	...	+	...	+	...
Ox, Blood Serum	...	...	...	...	...	o	...	...	...	...	...	...	...	...
Rabbit, Blood Serum	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Dog, Blood Serum	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Horse, Mixed Blood Corpuscles	...	...	...	...	...	+	...	...	+	...	+	+	...	...
Horse, Red Corpuscles	...	...	...	...	...	+	...	...	+	...	+	...	+	...
Dog, Sheep, Rabbit — Red Corpuscles	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Horse, Blood Platelets	...	...	...	...	...	+	...	...	...	...	...	...	...	...
„ Leucocytes	...	...	...	...	...	o	...	...	...	...	...	...	...	...
„ Plasma	o	...	...	o	o	o	...	...	+	o	+	o	+	+
„ Serum	o	...	...	...	o	...	...	...	+	...	+	o	+	+
Ox, Plasma	...	...	...	+	...	o	...	...	...	...	+	...	+	...
„ Red Blood Corpuscles	...	...	...	+	...	+	...	...	...	...	+	...	+	...
„ Blood Platelets	...	...	...	o or slight	...	o	...	...	...	...	o or slight	...	+	...

Glycyl-l-tyrosine is readily hydrolysed by trypsin, but not by pepsin, and it therefore serves an excellent compound for determining whether a given proteolytic ferment behaves as a peptic or a tryptic enzyme. For this reason it was employed by Abderhalden and Rona to determine the nature of the enzymes contained in the pyloric and duodenal

juices. Since neither of these juices acted upon glycyl-l-tyrosine they must be regarded as peptic in their nature.

Abderhalden and Teruuchi used glycyl-l-tyrosine to determine the nature of the enzymes in yeast juice, *i.e.* endotryptase, in papain and in the juice of nepenthes. The two former hydrolysed it, and consequently they contain tryptic enzymes; the last had no action upon it, and the enzyme of nepenthes is therefore like pepsin in its action. These results confirm the observations of other investigators, and the confusion concerning the nature of these enzymes would appear to be now settled with certainty.

The proteoclastic enzymes occurring in the germinating seeds of wheat and the lupine appear, according to the results obtained by Abderhalden and Schittenhelm, to have a stronger hydrolytic action than trypsin, since they break up glycyl-glycine and dl-leucyl-glycine which are unaffected by the enzyme of the pancreas. The same holds good for the enzyme of the mushroom, although glycyl-l-tyrosine was not hydrolysed; this compound was apparently destroyed by other enzymes in the mushroom.

The enzymes of the various organs of the animal body have hydrolysed with few exceptions all the polypeptides upon which their effect has been studied. Leucyl-leucine was not hydrolysed by the enzymes of the liver of the ox, and in all probability this was due to the insolubility of the polypeptide. The other striking result is that glycyl-l-tyrosine was not hydrolysed by the enzymes of calf's brain, which attacked the other polypeptides upon which it acted. The only diketopiperazine so far investigated was glycine anhydride, and this was not converted into glycine; this result would point to the absence of anhydrides in the products absorbed from the intestine. In general, the enzymes of the organs are more powerful than trypsin and less selective in their action.

The most interesting and astonishing facts were obtained by the examination of the blood corpuscles, the plasma and serum. Red blood corpuscles and platelets of the horse (but not of the ox) hydrolysed glycyl-l-tyrosine, which was not attacked by white corpuscles obtained from lymph or from pus cells, nor by the plasma or serum. Plasma and serum both hydrolysed dl-alanyl-glycine, as also the tri- and tetra-peptides diglycyl-glycine and triglycyl-glycine, which proves that the enzymes in the plasma and serum are not trypsin (or erepsin) absorbed from the intestine. Red blood corpuscles hydrolysed diglycyl-glycine, and the splitting caused by the plasma and serum may be due to the presence of the enzymes of the red blood corpuscles, either excreted naturally, or produced during the separation of the constituents, which

was not probably quite perfect, since it is well known that there are great difficulties to be surmounted in obtaining serum or plasma absolutely free from the red colour of the corpuscles.

On account of these results with serum Abderhalden and Rona investigated the action of human blood serum on glycyl-l-tyrosine in certain cases of disease, as also the urine. In some diseases no hydrolysis occurred, but in other diseases there was distinct hydrolysis. As yet no conclusions can be drawn from these results, as they require amplification both as regards the enzyme solution and the substrate. In no case had the urine any action upon glycyl-l-tyrosine; this seems at variance with the presence of an urotryptic enzyme which Cathcart studied in its action upon proteins.

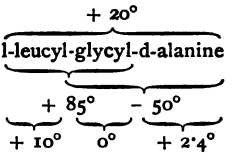
The hydrolysis of polypeptides by enzymes shows most conclusively that the protein molecule is built up of amino acids combined together in the form of acid amides, but there still remains the possibility that other modes of combination are present. In the animal body, proteins are acted upon firstly by pepsin, then by trypsin and then by the other enzymes. Pepsin does not hydrolyse any of the polypeptides, but it hydrolyses the proteins producing a mixture of some five or six proteoses and peptones. Trypsin hydrolyses the majority of the polypeptides, and it hydrolyses the proteins producing amino acids, together with a complex polypeptide, as Fischer and Abderhalden have shown, which contains all the proline and all the phenylalanine which are present in the protein. This complex polypeptide occurs in a modified form when a protein is hydrolysed first by pepsin and then by trypsin; some of the proline and the phenylalanine are obtained in the free state. The complex polypeptide is hydrolysed by the various intracellular enzymes in the organs of the body, since it is not excreted. These enzymes hydrolyse polypeptides which are not attacked by trypsin; such combinations are therefore probably contained in this complex. The enzymes in the various organs are extremely diverse in their nature: certain purine bases are acted upon by the enzymes of one organ but not by those of another organ, the arginase of the liver hydrolyses arginine (Kossel and Dakin), but not the similarly constituted creatine (as Dakin has recently shown). Enzymes are characterised by their highly selective nature; they act upon one definite substance or upon groups of substances, *e.g.*, the fats and the polypeptides. Pepsin does not act upon the polypeptides, but it hydrolyses the proteins; either the polypeptides are not sufficiently complex to be attacked by pepsin, or pepsin acts upon another mode of combination of the amino acids. One

mode of combination other than a polypeptide linking is present in arginine, and other modes are still possible. Bergell and Feigl have prepared combinations where two amino acids are combined to the ammonia by both their carboxyl groups, and these are not attacked by trypsin nor by pepsin. The oxyamino acids may be combined in the form of ethers with one another, or in the form of esters with other amino acids, and anhydrides of amino acids are possible. Further, diketopiperazine rings may occur, certain of which are easily hydrolysed by alkali.

Proteins, according to Cohnheim, are not hydrolysed by erepsin, the enzyme of the intestinal mucous membrane, which hydrolyses only the proteoses and peptones, converting them into amino acids; if proteins are previously hydrolysed by pepsin, they are then converted into amino acids by erepsin. Pepsin would therefore appear to have a special function rather than act like trypsin and the other enzymes, and it may attack one of the other possible linkings of amino acids. If it only produces some five or six products, there would only be the same number of such linkages.

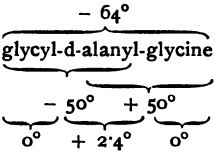
The optically active dipeptides, d-alanyl-d-alanine and d-alanyl-l-leucine, have been employed by Abderhalden and Koelker for comparing the activity of various enzymes, such as pancreatic trypsin, yeast endotryptase and intestinal erepsin; by observing the change in rotation they were able to determine the rate at which these polypeptides were hydrolysed, and they found that yeast endotryptase was the most active, erepsin attacked the dipeptide more slowly, and trypsin in forty-eight hours had scarcely hydrolysed it at all. Not only can the rate of change in rotation, which property was made use of for this purpose, be used to show differences in the various enzymes, but also it can be used for determining the rate of action (see monograph by W. M. Bayliss, F.R.S., on Enzyme Action) of the enzyme under various conditions as Abderhalden, in conjunction with Michaelis and Gigon, has shown.

By means of the change in rotation Abderhalden and Koelker have also attempted to determine at which point a tripeptide is first attacked by an enzyme. The specific rotation of l-leucyl-glycyl-d-alanine is + 20°, that of l-leucyl-glycine is + 85°, and that of glycyl-d-alanine is − 50°. An increase in rotation would show that d-alanine was first separated and l-leucyl-glycine formed; a decrease in rotation would point to a separation of l-leucine and the formation of glycyl-d-alanine according to the following scheme :—



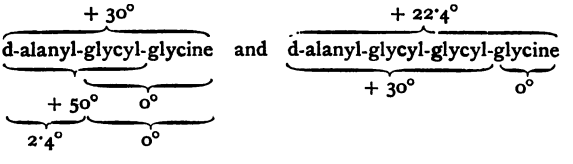
It was observed that the rotation at first increased to an extent of about 40 per cent., and under these conditions l-leucyl-glycine must be first formed and alanine separated off. Later, the rotation decreased which was due to the hydrolysis of l-leucyl-glycine. Glycyl-d-alanine was apparently not formed at all in the process of hydrolysis.

The tripeptide glycyl-d-alanyl-glycine was also investigated and the point of first attack determined. The rotations of the various compounds are :—



In the experiment the rotation decreased at first, was reversed in direction and then again decreased in amount. Glycine and d-alanyl-glycine must therefore have been formed first and the d-alanyl-glycine must then have been subsequently hydrolysed.

Further experiments were shortly afterwards made upon the compounds,



but here yeast endotryptase was also employed. By this enzyme hydrolysis was effected in such a way that the rotation in both cases gradually decreased which showed that d-alanine was first separated.

The action of trypsin upon d-alanyl-glycyl-glycine was also determined. The hydrolysis took place differently; the rotation decreased a little, increased considerably and then again decreased. This shows that glycine is first separated by trypsin, with the formation of d-alanyl-glycine, whereas endotryptase first split off d-alanine.

On account of this extraordinary difference the action of endotryptase upon l-leucyl-glycyl-alanine was examined. The rotation decreased and became negative, which showed that this tripeptide was completely

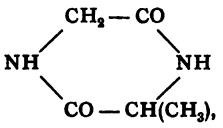
split open between the leucine and the glycine. With trypsin l-leucyl-glycine was first formed and d-alanine split off. We have thus a difference in the point of attack of enzymes of different origin, and may therefore possess a sharp means of differentiating between the various proteoclastic enzymes; it may prove of use in determining the nature of a complex polypeptide resulting by the hydrolysis of protein.

The Polypeptides Present in the Proteins.

The formation of a dipeptide by the hydrolysis of silk-fibroin was first described by Fischer and Bergell in 1902.

As is well known, silk-fibroin readily dissolves in cold concentrated hydrochloric acid ; if alcohol be then added, a product, called sericoin by Weyl, is precipitated, but if the silk-fibroin be allowed to stand in contact with three times its quantity of concentrated acid for about twenty-four hours, alcohol no longer produces such a precipitate, and the solution contains the hydrochloride of a peptone. On concentration in vacuo, when freed from hydrochloric acid, a mass was obtained which had a bitter taste, was very soluble in water and gave strong biuret and Millon reactions, and which was very like peptone in its properties. When dissolved in water and digested in ammoniacal solution with trypsin, this peptone lost the whole of the tyrosine which it contained, and was converted into another peptone composed of 40·1 per cent. glycine and 28·5 per cent. alanine. From this compound, when heated with baryta water, ammonia was evolved, and the solution, freed from baryta, on evaporation yielded crystals; these were treated with β-naphthalene-sulphochloride, and a compound was obtained which was apparently β-naphthalene-sulpho-glycyl-alanine, though it could not be absolutely identified with the synthetical product of this composition.

The further attempts to again prepare this substance did not succeed, since the exact conditions leading to its formation could not be repeated, but in 1906 Fischer and Abderhalden obtained the anhydride of this body by a new method which they had discovered for isolating such compounds when mixed with amino acids and higher polypeptides. This method depends upon the different behaviour of the esters of these compounds; those of the simple mono-amino acids are easily volatile in vacuo and are therefore easily removed, whereas those of the dipeptides are converted by the action of ammonia into their anhydrides or diketopiperazines which crystallise readily and are therefore easily separated from the esters of the higher polypeptides. They thus obtained a methyl diketopiperazine,



which was identical with a synthetical product prepared from glycine and d-alanine, and which yielded glycine and d-alanine on hydrolysis.

It resulted by the hydrolysis with 70 per cent. sulphuric acid followed by trypsin, and by the hydrolysis with hydrochloric acid.

This diketopiperazine could arise both from glycyl-d-alanine and d-alanyl-glycine by the above method. It could also arise by synthesis from glycine and d-alanine under the conditions of the experiment; control experiments were carried out to determine this possibility, and they showed that this was impossible so that there was no doubt concerning the presence of a dipeptide amongst the products of hydrolysis. Since this dipeptide was resistant to trypsin it was most probably glycyl-d-alanine and not d-alanyl-glycine which is easily hydrolysed by this enzyme.

At the same time another diketopiperazine, glycyl-l-tyrosine anhydride, was also obtained; its nature was established a little later by identification with synthetical glycyl-l-tyrosine anhydride prepared from the ester of chloracetyl tyrosine and ammonia. In one experiment its yield amounted to 4·2 per cent. of the silk-fibroin employed.

In the same way by the hydrolysis of elastin with 70 per cent. sulphuric acid and by the action of ammonia upon the esters, a product was isolated which was composed of glycine and l-leucine and which was identical with synthetical glycyl-l-leucine anhydride.

In 1907 Fischer and Abderhalden definitely showed that the first diketopiperazine isolated from the hydrolysis products of silk-fibroin was derived from glycyl-d-alanine. Silk-fibroin was partially hydrolysed by hydrochloric acid and then precipitated by phosphotungstic acid. A portion of the filtrate from this precipitate, after removal of the excess of phosphotungstic acid, was treated in alkaline solution with β-naphthalene-sulphochloride and a product was obtained which was identical with β-naphthalene-sulpho-glycyl-d-alanine; further proof of this was given by its careful hydrolysis with dilute hydrochloric acid when the dipeptide chain was split, but the naphthalene-sulpho radical not removed; β-naphthalene-sulpho-glycine and d-alanine were obtained according to the equation:—

$$C_{10}H_7SO_2 — NH . CH_2 . CO — NHCH(CH_3)COOH + H_2O =$$
$$C_{10}H_7SO_2 — NH . CH_2 . COOH + NH_2 . CH(CH_3) . COOH$$

From the remainder of the filtrate, glycyl-d-alanine anhydride and a small quantity of glycyl-l-tyrosine anhydride were obtained by the action of ammonia upon the esters, as well as another product which was most probably d-alanyl-l-serine anhydride.

An examination of the phosphotungstic acid precipitate showed that it contained several products of a complex nature. From them a substance was isolated which consisted of two molecules glycine, one

molecule alanine, and one molecule tyrosine, *i.e.*, a tetrapeptide. It had a molecular weight determined by the freezing-point method of about 350, was easily soluble in water, insoluble in alcohol, and was precipitated from its solution in flakes by saturation with ammonium sulphate or sodium chloride, as also by nitric or acetic acid. The synthetical pentapeptide l-leucyl-triglycyl-l-tyrosine behaves in a similar way so that great complexity, as formerly believed, is not an essential condition for precipitation by ammonium sulphate. By the action of trypsin tyrosine was split off, and on partial hydrolysis glycyl-d-alanine anhydride and glycyl-l-tyrosine anhydride were obtained.

In the same year (1907) the products obtained from elastin by partial hydrolysis were shown by Fischer and Abderhalden by the same methods to contain—

1. d-Alanyl-l-leucine. The anhydride of this dipeptide was also obtained; it probably arose from this dipeptide, but it can also be formed from the isomeric l-leucyl-d-alanine, whose presence amongst the products is not excluded.

2. Alanyl-proline anhydride, from which d-alanine and proline were obtained on hydrolysis.

3. Glycyl-valine anhydride, which was identical in its properties, except the melting-point, with the synthetical compound.

From gliadin Fischer and Abderhalden have also isolated a dipeptide by these methods, namely, l-leucyl-d-glutamic acid, which they identified with the synthetical substance; Abderhalden and Funk have isolated leucinimide, l-phenylalanyl-d-alanine anhydride by acid hydrolysis from casein, and probably also leucyl-valine anhydride.

In addition to these dipeptides isolated by Fischer and Abderhalden, Osborne and Clapp have obtained a crystalline substance by the acid hydrolysis of gliadin, which yielded proline and phenylalanine on further hydrolysis, and Levene and Beatty have isolated a dipeptide composed of glycine and proline from the products resulting by a trypsin digestion of gelatin. The exact nature of these bodies has still to be determined by comparison and identification with the synthetical substances, and not until this has been done can their presence in the molecule be accepted with certainty.

The appended list gives the polypeptides which have so far been isolated from proteins and therefore of the combinations of amino acids which occur in the protein molecule:—

Glycyl-d-alanine anhydride in silk-fibroin.
Glycyl-d-alanine in silk-fibroin.
Glycyl-l-tyrosine anhydride in silk-fibroin.

d-Alanyl-l-serine anhydride (?) in silk-fibroin.
Glycyl-l-leucine anhydride in elastin.
d-Alanyl-l-leucine anhydride in elastin.
d-Alanyl-l-leucine in elastin.
d-Alanyl-proline anhydride in elastin.
Glycyl-valine anhydride in elastin.
Leucinimide in caseinogen.
l-Phenylalanyl-d-alanine anhydride in caseinogen.
l-Leucyl-d-valine anhydride in caseinogen.
l-Leucyl-d-glutamic acid in gliadin.
Dipeptide (proline + phenylalanine) in gliadin.
Dipeptide (proline + glycine) in gelatin.
Tetrapeptide (2 glycine + 1 alanine + 1 tyrosine) in silk-fibroin.

It is only by the knowledge of the properties of the synthetical compounds that Fischer has been able to invent methods for isolating them from the mixtures which result by the hydrolysis of the proteins and to identify these compounds. The extension of the study of the higher polypeptides, more especially of the mixed polypeptides, will lead without doubt to the isolation of greater complexes from the products of partial hydrolysis of the proteins, such as the separation of the proteoses and peptones, which from the results so far obtained appear to be more simple than was previously supposed, if salting out by ammonium sulphate of polypeptides containing four and six units, of which tyrosine is one, be taken as a typical example.

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